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The Fifth Semiannual Progress Report on

ENZYME ACTIVITY IN TERRESTRIAL SOIL IN RELATION TO EXPLORATION OF THE MARTIAN SURFACE

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I. INTRODUCTION

Our objective is to develop qualitative and quantitative tests for various enzyme activities in soil and to adapt the most sensitive and suitable of these to procedures compatible with telemetry from Mars probes. In addition, we are examining enzyme reactions in environments of limited water availability and at interfaces.

In view of the emerging interest in the studies of free enzymes in soils, an attempt was made to locate the microsites of soil phosphatase activity with electron microscopic methods.

The Martian environment has a limited moisture contact and any biological reactions possibly take place at interfaces and on surfaces in an environment of restricted water availability. A study of surface effects in the hydrolysis of insoluble chitin by adsorbed chitinase and lysozyme is being continued in order to investigate some of the factors influencing reactions at interfaces.

Studies of urea hydrolysis by urease in media of low water availability were continued. The reaction is detectable at 65% relative humidity and the method is being developed further for its possible use for the detection of catalytic, i.e., enzymatic, breakdown of urea in Martian environment. Examination of the detection of urease activity has been selected because of the probable primordial origin of urea as an organic substance, because of its relative stability as an enzyme substrate, and because of the ubiquity of soil urease in the terrestrial environment.

There exists a possibility that extraterrestrial microorganisms

can metabolize hydrocarbons (if present from an abiotic synthesis) and use them as an energy and/or carbon source. Present knowledge of the microbial metabolism of alkane hydrocarbons is scanty, and the initial steps of their metabolic degradation are uncertain. Therefore, we have initiated a study to examine the initial metabolic steps of hydrocarbon degradation by terrestrial microorganisms.

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II. EXPERIMENTAL

A. Localization of Phosphatase Activity in Soils.

The localization of enzyme activity in soils has been the subject of a wide range of speculation. Thus, the fact that most of the attempts to extract enzymes from soils have been unsuccessful is taken by some workers in the field as evidence that these enzymes are located solely within the microbial cells.

Other workers have suggested that enzymes accumulate in soils, but disagree about their sources. The adsorptive properties of soils, the absence of marked fluctuations in soil enzyme activity, and the lack of correlation between enzyme activity and the microbial population when the same soil is sampled at different times of the year, are facts usually offered, either together or separately, in support of the hypothesis of an accumulation of extracellular enzymes in soils. However, no direct evidence has ever been presented on the localization of enzyme activity in soils.

The soil clays and the clay minerals have been the object of numerous studies by means of the electron microscope, but only a few of the studies have been concerned with the fine structure of soils, and with the relationships among roots, microorganisms and soil particles. This subject was thoroughly reviewed, recently, by Egawa and Watanabe (4). Studies of the root-soil boundary made by Jenny and Grossenbacher (11), and the technical developments on enzyme histochemistry at the electron microscope level (5, 10),

stimulated this work on the ultrastructural localization of phosphatase activity in soils. Since whole soil contains particles which are very hard to section even with the aid of a diamond knife, the possibility of using a particular fraction of the soil showing a high phosphatase activity was considered the best choise for embedding and sectioning purposes. Water-soluble resins and mixtures of them with other resins were thought to be ideal for embedding purposes, according to the results reported by Leduc et al. (17) and Leduc and Holt (16) on the extraction of proteins and nucleic acids from biological specimens by direct treatment of ultrathin sections with the corresponding enzymes in solution.

Materials

Soils. Fresh surface samples from Oxford Tract loam soil and air-dried samples from Dublin clay loam soil were used. Characteristics of these two soils are given in Table 1.

Table 1

Characteristics of Soils

Soil Type	Organic C as %*	pH (1:1)	Mechanical analysis as %** <2μ	2-20μ	>20μ
Dublin clay loam	2.74	5.6	29	25	46
Oxford Tract loam	2.83	6.7	20	33	47

* Dry combustion method

** Soil fractions extracted in their natural state by sedimentation at 3°C.

Clays. Peerless kaolinite and Wyoming bentonite were prepared as described by McLaren (23) and McLaren et al. (24).

Substrates. Na-β- glycerophosphate from Fisher Scientific Co., Fair Lawn, N. J., and Na-β- naphthylphosphate from Calbiochem, Los Angeles, Calif., were used.

Enzyme. Wheat acid phosphatase was obtained from Worthington Biochemical Corp., Freehold, N. J.

Buffers. Modified universal buffer (MUB) as described by Skujins et al. (28) was used. Additionally, 0.2 M acetate and 0.2 M Tris-maleate buffers were used.

Embedding resins used:

Epon 812 (Shell Chemical Co., N. Y.);

ethylene glycol monomethacrylate (GMA) (Monomer-Polymer Laboratories, Philadelphia, Penn.);

methyl- and butylmethacrylate (Matheson Coleman and Bell, Norwood, Ohio);

hydroxypropyl methacrylate (HPMA) (Rohm and Haas, Philadelphia, Penn.).

Hardeners and catalysts used:

Dodecenyl succinic anhydride (DDSA) and nadic methyl anhydride (NMA) (General Chemical Division, Allied Chemical and Dye Corporation, N. Y.);

benzoyl peroxide (Eastman Organic Chemicals, Rochester, N. Y.);

ammonium persulphate (J. R. Baker Chemical Co., Phillisburg, N. J.).

Culture Media

a. Yeast-extract nutrient agar (YEN-agar) was prepared as follows:

beef extract	3 g
Bacto-pectone	5 g
Yeast extract	5 g
agar	15 g

The ingredients were dissolved in one liter of distilled water and the pH was adjusted to 7.0 by adding KOH. The medium was autoclaved for 15 minutes.

b. Tryptone yeast-extract broth (TYE-broth) was prepared as as described by Loeb and Zinder (18).

c. "Minimal medium-TEA" was prepared as described by Malamy and Horecker (20).

Methods

Fractionation by size of Dublin and Oxford Tract soils. 200-gram portions of native Dublin and Oxford tract soils were placed into 2-liter glass bottles and suspended in one liter of distilled water. The soil suspensions were shaken for 4 hours. Then another liter of distilled water was added to each bottle and the soil suspensions were stored at 3°C overnight.

For fractionation purposes the soil particles were classified according to size in the following way:

a. Clay fraction: particles less than 2μ in diameter.

b. Silt fraction: particles between 2 and 20μ in diameter.

c. "Course fraction": particles above 20μ in diameter.

Fractionation of the soil particles took place by sedimentation at 3°C. Stoke's law was used to calculate the time after which particles of a size below the upper limit of clay and silt fractions were still suspended in a given column of soil suspension. In these calculations, a density of 2.4 g/cu. cm was used for soil particles. In fractionation by sedimentation it is assumed that soil disperses completely in water and that all soil particles settle as if they were smooth and rigid spheres.

The clay fraction was siphoned out from the sedimentation bottle after completion of the time required to leave in suspension only those particles less than 2μ in diameter. The remaining soil material was suspended in distilled water at 3°C and allowed to sediment again. Clay collection was repeated until the supernatant fluid of the soil suspension was almost clear after the sedimentation period. The silt fraction was collected using a procedure similar to that described above for the clay fraction. The soil material remaining after the silt collection was designated as the "coarse" fraction. The clay and silt fractions were concentrated by means of a candle-shaped, Selas bacteriological filter connection to a vacuum system.

Phosphatase activity of Dublin soil fractions. The fluorimetric assay previously described (30) was used to determine the phosphatase activity not only of one-gram samples of soil fractions, but also of aliquots of the supernatant fluid in which soil fraction was kept in the form of pellets after extraction. Phosphatase activity determinations were carried out at a temperature of 35°C.

Microbial numbers of Dublin soil fractions. Suspensions containing one gram per 10 ml of clay and of silt fractions were prepared by suspending the corresponding pellets in sterile distilled water. From these two stock suspensions, a dilution series was prepared with the aid of sterile distilled water. A 2-ml portion of the supernatant fluid corresponding to 7.77 g of the clay fractions, and a 4-ml portion of the supernatant fluid corresponding to 14.27 g of the silt fraction were diluted to 10 ml with sterile distilled water. From each of these two stock solutions a dilution series was prepared

as indicated above. One-ml aliquots of the dilutions of either soil fractions or their supernatant fluids were plated on YEN-agar.

Embedding of soil clay and of clay-phosphatase complex in water-soluble resins. A bentonite-phosphatase complex was prepared by the same procedure previously described (31) for the preparation of the kaolinite-phosphatase complex. Soil clay and bentonite-phosphatase complex pellets (approximately 50 mg dry weight), were fixed in 6.5% (v/v) glutaraldehyde for one hour at 4°C. After this, the soil clay pellets were washed three times with Tris-maleate buffer (pH 7.0), while the bentonite-phosphatase pellets were washed three times with acetate buffer (pH 5.0). Fixed and unfixed pellets of both soil clay and bentonite-phosphatase complex were dehydrated step-wise by suspending them in mixtures of water with either GMA or HPMA as described by Leduc and co-workers (16, 17), according to the following schedule:

GMA	HPMA
a. 80% of 97% GMA	a. 80% of 97% HPMA
b. 97% GMA	b. 97% HPMA
c. 1:1 mixture of 97% GMA with the "Leduc" medium	c. Prepolymer 97% HPMA

In each case, separation of the impregnated soil clay and bentonite-phosphatase complex from the dehydrating fluid was carried out by sedimentation in a refrigerated centrifuge. The 97% GMA solution contained 0.5% of ammonium persulphate, while the 97% HPMA solution contained 0.1% of azonitrile. The stabilizer was removed

from the monomeric GMA by vacuum distillation before adding the ammonium persulphate. HPMA was not destabilized.

Polymerization followed after one change of the final embedding medium. For GMA the final embedding medium consisted of a 1:1 mixture of the "Leduc" medium (70% of 97% GMA and 30% of 15/85% butyl-/methylmethacrylate plus 1% benzoyl peroxide) and Luft's complete resin mixture (18), as described by McGee-Russell and de Bruijn (22). Polymerization of the GMA mixture took place in less than 24 hours in the presence of light at 37°C. For HPMA the final embedding medium was 97% HPMA. Polymerization of HPMA was induced by exposing the capsules to long wavelength UV light (above 3000 Å) from a Hanovia utility lamp No. 30600 provided with a Pyrex filter. Polymerization of HPMA was completed in less than 24 hours at 3°C.

Ultrastructural localization of phosphatase activity of soil clays and of clay-phosphatase complexes. The Gomori substrate solution (6) was used for the demonstration of phosphatase activity at the ultrastructural level in soil clays and in clay-phosphatase complexes. The Gomori substrate solution was prepared as follows: 0.6 g of either lead nitrate or lead acetate were dissolved in 500 ml of either 0.05 M acetate or 0.05 M Tris-maleate buffer. Lower and higher concentrations of lead salts were also used. To any of the resulting solutions 50 ml of a fresh 3% (w/v) solution of Na-β-glycerophosphate were added. The final solutions were left at 4°C overnight and then were filtered through a 0.22μ pore-size Millipore filter. The substrate solution was adjusted to pH 5.0 for the clay-phosphatase complex, and to pH 7.0 for the soil clay.

Phosphatase reaction of ultrathin sections. The resin blocks obtained after polymerization of both the GMA and the HPMA preparations containing either soil clay or bentonite-phosphatase complex, were trimmed to give a cutting face with edge length of 20-100μ. The trimmed resin blocks were mounted on a L.K.B. Untratome provided with a diamond knife. Sections 200-600 Å units in thickness were cut with the diamond knife and were either mounted on carbon-coated gold grids or transferred directly, by means of the ring technique developed by Marinozzi (21), to two containers of the Gomori solution: one with the substrate, the other without it. The carbon-coated grids holding the mounted sections were also floated upside-down on the Gomori solutions. The phosphatase reaction was allowed to take place for periods of time ranging from 30 minutes to 16 hours. Afterwards the sections were washed two or three times by floating them on distilled water. Sections were examined in an EMU 3 electron microscope.

Phosphatase reaction of soil clay suspensions. The technique usually employed when dealing with unicellular specimens (3, 29) was followed for the determination of the site of phosphatase activity in soil clay suspensions. Briefly, the soil clay was first fixed with glutaraldehyde and then incubated for given periods of time in buffered solutions containing or lacking glycerophosphate, using the incubation procedure utilized for the determination of phosphatase activity in soils as described (30). Afterwards the clay material was washed more than five times to rid it of unreacted substrate and lead nitrate. Finally, the material was embedded in a GMA-Epoxy

resin mixture as described above. Sections were cut with the diamond knife and observed in the electron microscope.

Phosphatase reaction of soil clay and of clay-phosphatase complexes spread on a carrier film. To spread the material to be tested for phosphatase activity on the carrier film, a small chromatography-like trough was filled completely with glass distilled water, and, after making sure that the surface of the water was free of contaminating material, a glass rod wet with the suspension of either soil clay or clay-phosphatase complex was allowed to come in contact with the surface of the water. Part of the material adsorbed to the glass rod floated and spread on the water forming a discontinuous film. Gold grids coated with formvar film were then floated on those places where the material was seen floating. The formvar film was made by the technique described by Sadvir (27), but using mica leaves, split just before being used, instead of glass slides. The electron microscope grids were picked up and immediately floated again on a buffered solution either containing the substrate or lacking it. After a given period of time was allowed for the reaction to take place, the grids were removed from the buffered solution and floated on distilled water for no less than 12 hours. The grids were shadowed with uranium and/or covered with a very thin film of carbon. The film of carbon provides stabilization of the particulate material.

Phosphatase reaction of soil bacteria spread on a carrier film. A bacterium particularly active in hydrolyzing phenolphthalein phosphate was isolated from Oxford Tract soil by using the technique

described by Kotelev (13). The bacteria were allowed to grow in TYE-broth for 4 days. Afterwards one-ml portions of the bacterial culture were removed and sedimented at 5,000 rpm. The resulting pellets were resuspended in "minimal medium-TEA" deficient in inorganic phosphate. After a three-hour derepression treatment with the minimal medium, the bacterial suspensions were sedimented again. The bacterial pellets were fixed with glutaraldehyde, as previously described, and then washed twice with Tris-maleate buffer (pH 7.0). Formvar-coated grids were floated on one drop of bacterial suspension and then transferred to a Gomori solution containing or lacking the substrate. After incubation for 20 hours, the grids were washed twice by floating them on distilled water for more than 12 hours each time. The results were observed in the electron microscope.

Results

Phosphatase activity and microbial numbers of Dublin soil fractions. Table II shows the distribution of phosphatase activity and microbial numbers among Dublin soil fractions. In the clay and silt fractions phosphatase activity per unit weight was approximately the same, whereas in the "coarse" fraction it was approximately one half of the activity shown by either the clay or the silt fraction. The supernatant fluids of the clay fraction and of the silt fraction accounted for only 0.8% and 0.2% respectively, of the phosphatase activity of whole soil. If the contributions of all of the soil fractions are added up, they account for 83% of the phosphatase of the whole soil.

With regard to microbial numbers, it was observed that the

TABLE II

Phosphatase Activity and Microbial Numbers of Dublin Soil Fractions

Sample designation	Contribution to the total weight of whole soil as %	β-naphthol moles extracted/hr/g of soil fraction	Bacterial colonies per g of soil fraction (millions)	Contribution to the total activity of whole soil as %
Clay fraction	29	6.44	18	31
Clay fraction supernatant fluid	-	0.16	2	0.8
Silt fraction	25	6.39	2	27
Silt fraction supernatant fluid	-	0.05	3.3	0.2
"Coarse" fraction	46	3.12	-	24
Whole soil	100	6.0	-	100

supernatant fluid of the silt fraction, which had less than 1% of the phosphatase activity of the silt fraction, showed a higher bacterial population than the silt fraction itself. On the other hand, although the clay and silt fractions showed the same level of phosphatase activity, the bacterial population in the clay fraction was nine times larger than in the silt fraction.

Ultrastructural localization of phosphatase activity in soil clays and in clay-phosphatase complexes. The photographs appearing in the upper part of Figure 1 pertain to the phosphatase reaction carried out on sections of bentonite-phosphatase complex embedded in HPMA. Sections incubated for 4 hours in the Gomori medium containing the substrate (upper left photograph) did not differ from those sections incubated for the same length of time in the Gomori medium lacking the substrate (upper right photograph). This was also the case for sections of clay soil embedded in either HPMA or GMA-Epoxy resin mixture.

The lower part of Figure 1 shows the results of carrying out the phosphatase reaction in Oxford Tract soil clay suspension. The clay suspension was incubated for 45 minutes with the Gomori solution either containing or lacking the substrate. The soil clay suspension treated with the Gomori solution containing glycerophosphate (lower left photograph) shows the deposit of numerous black, crystal-like structures. These same structures are present, also, in the corresponding control (lower right photograph), but they are less numerous.

Figure 2 represents the results of the phosphatase rection of

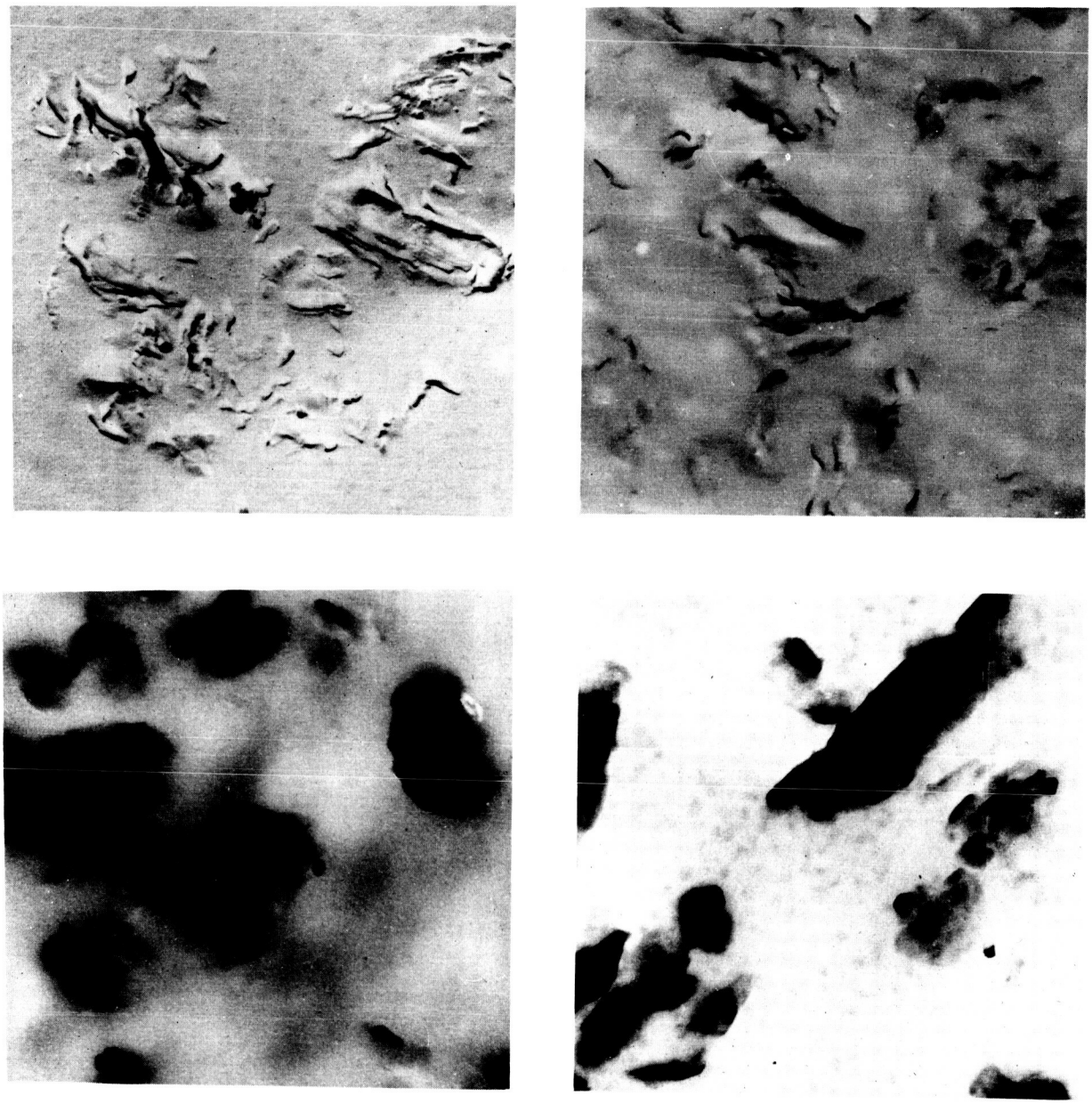


Figure 1. Phosphatase reaction of bentonite-phosphatase sections (upper), and of soil clay suspensions (lower). Incubation in the Gomori medium containing (left) or lacking (right) the substrate.

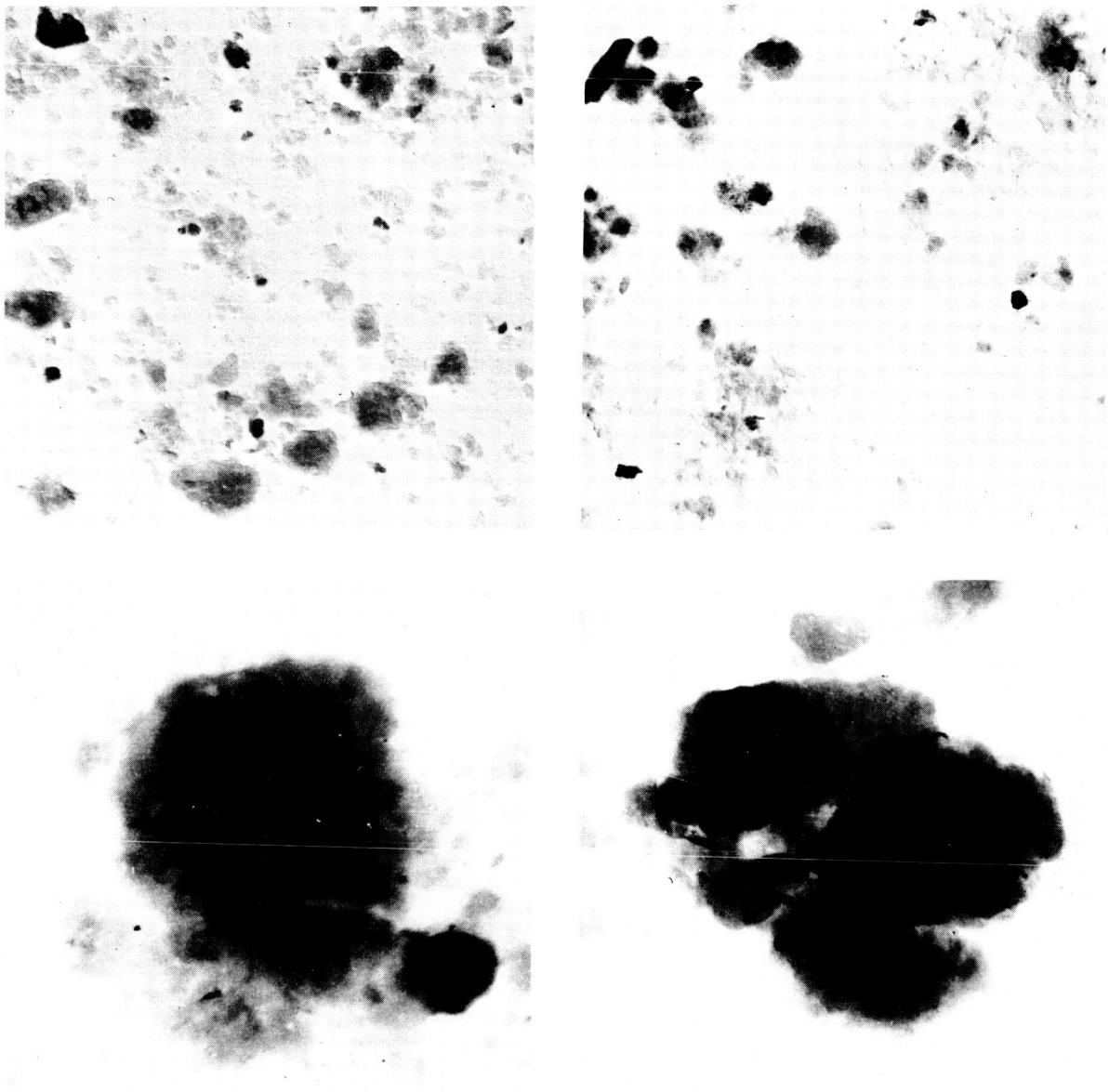


Figure 2. Phosphatase reaction of soil clay spread on a carrier film. Incubation in the Gomori medium containing (left) or lacking (right) the substrate.

soil clay spread on a carrier film. The reaction was allowed to take place at room temperature overnight (16 to 20 hours). It can be seen that there is no difference between the clay preparation treated with the Gomori solution containing the substrate (upper left photograph), and the corresponding control (upper right photograph). Occasionally, some soil clay particles treated with the complete Gomori medium presented darkened areas on their surface (lower left photograph). The darkened areas were rarely seen in soil clay particles treated with the incomplete Gomori medium lacking the substrate (lower right photograph).

The results on the phosphatase reaction of bentonite-phosphatase and kaolinite-phosphatase complexes spread on a carrier film were not different from those described above for soil clay. Figure 3 shows the bentonite-phosphatase (upper left) and kaolinite-phosphatase (upper right) complexes spread on a carrier film after incubation with the complete Gomori medium. No controls are included because they were indistinguishable from the glycero-phosphatase treated samples. Figure 3 also shows two more photographs (lower part) of clay soil spread on a carrier film after incubation with the complete Gomori medium.

Figure 4 shows how bacteria present in soil clay spread on a carrier film reacted toward the Gomori medium either containing or lacking the substrate. In the presence of the substrate (left side photographs) bacteria were slightly darker than in its absence (right side photographs). Otherwise, they were completely unchanged. These bacteria were not intentionally stained.

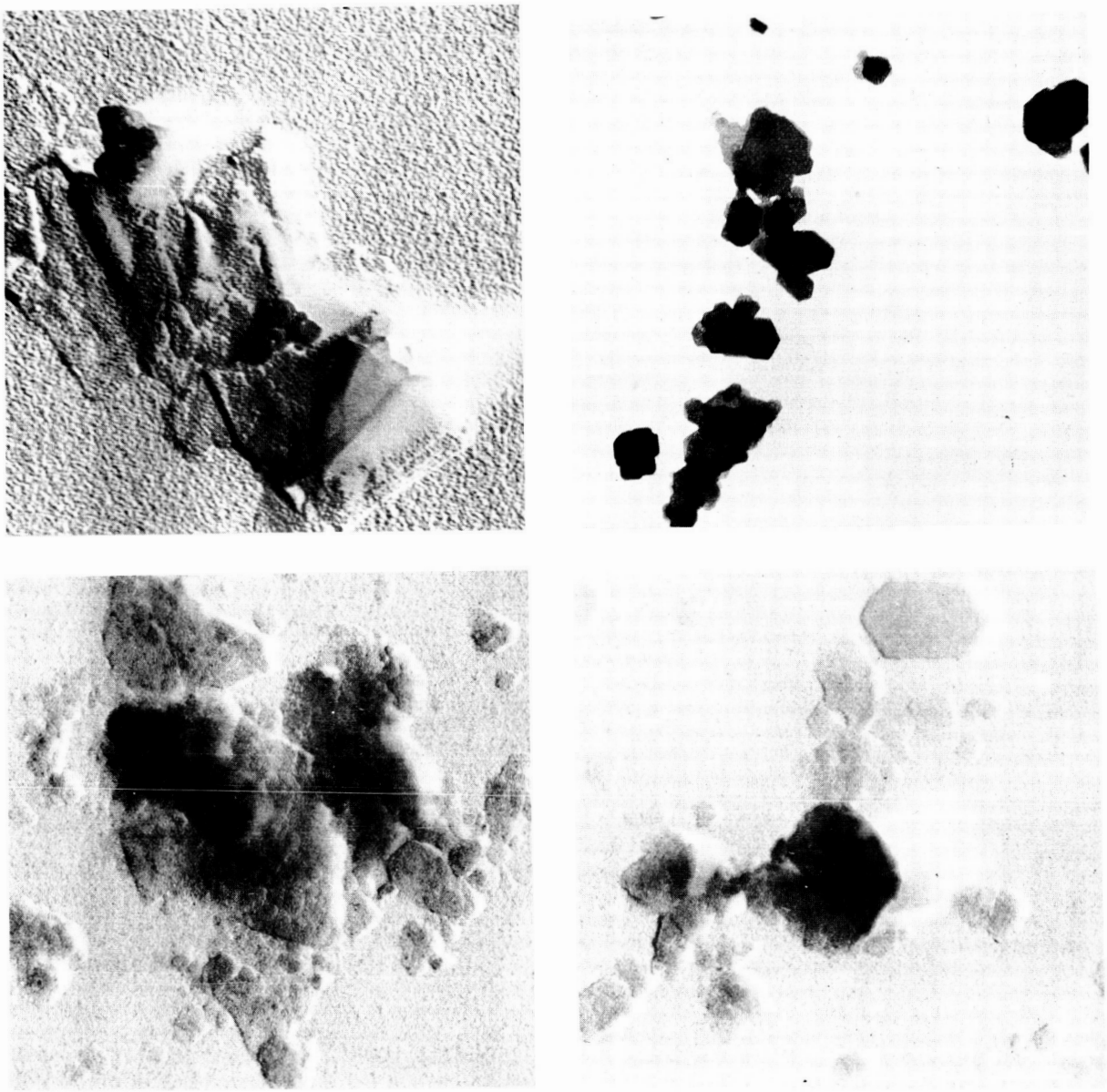


Figure 3. Phosphatase reaction of bentonite-phosphatase (upper left) and kaolinite-phosphatase (upper right) complexes, and of soil clay (lower) spread on a carrier film. Incubation in the Gomori medium containing the substrate.

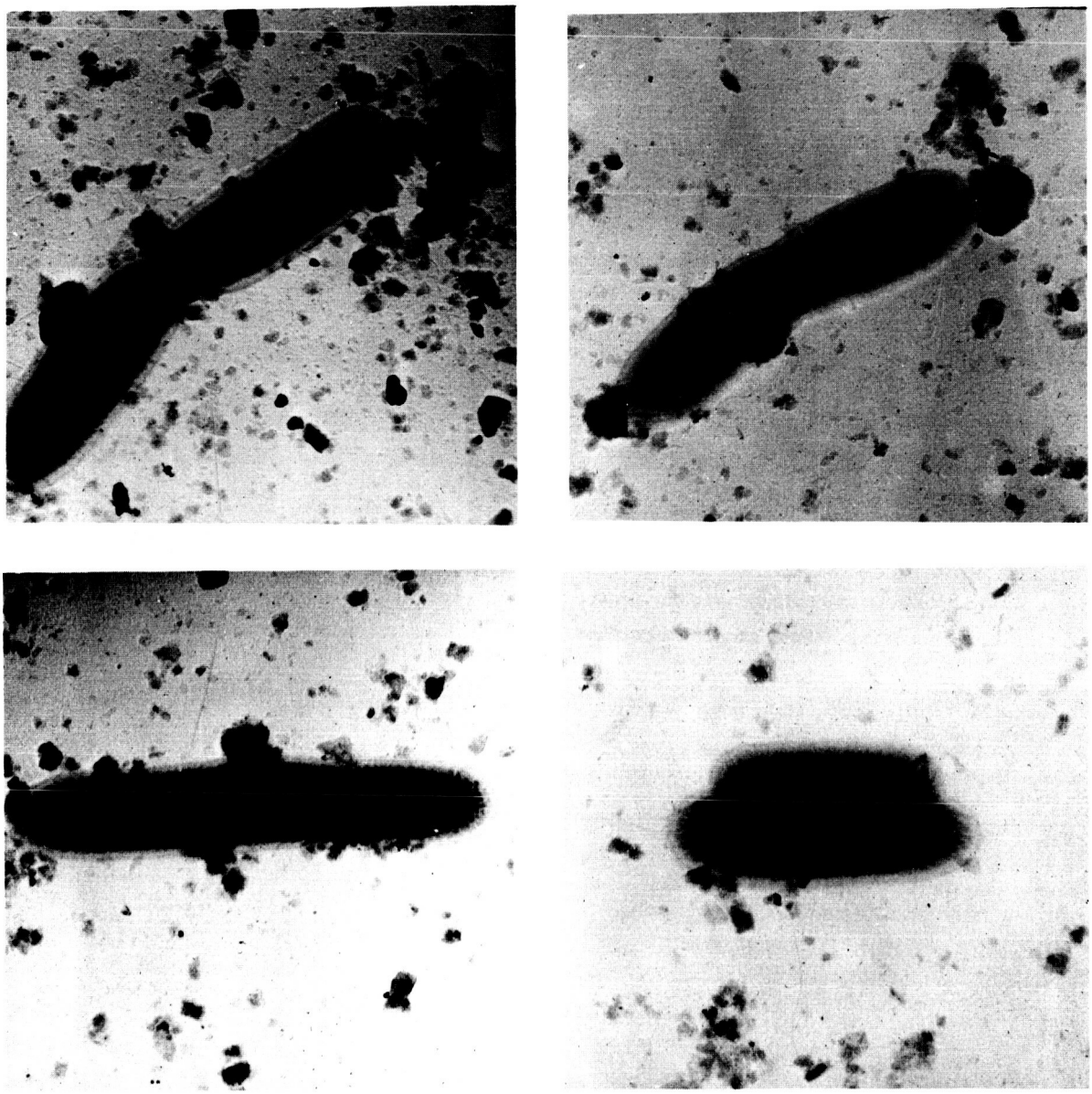


Figure 4. Phosphatase reaction of bacteria present in soil clay spread on a carrier film. Incubation in the Gomori medium containing (left) or lacking (right) the substrate.

The results shown in Figure 5 were obtained when a bacterium isolated from Oxford Tract soil for its ability to hydrolyze phenolphthalein phosphate, was examined for phosphatase reaction. Photographs on the left side show that intact bacteria incubated for 20 hours with the complete Gomori medium were also slightly darker than those intact bacteria incubated with the control medium (right side photographs). In the presence of the substrate, intact bacteria also showed the presence of black dots (left side photographs). These black dots were completely absent from intact bacteria incubated with Gomori medium lacking the substrate (right side photographs). Disrupted bacteria, which were more abundant than intact bacteria, could not be differentiated from each other when incubated in the Gomori medium either containing or lacking the substrate. They always appeared as elongated structures with broken ends and heavily stained, as is seen in the two middle bacterial cells shown in the lower right of Figure 5. It was not determined whether the disruption of bacterial cells was due to either the pre-treatment handling or the long incubation in the presence of lead salts.

Figure 6 shows soil clay embedded in GMA-Epoxy resin mixture. In the untreated sections (upper photographs) it can be observed that no voids are present between the clay particles and the surrounding embedding material. When the sections in the upper photographs of Figure 6 were stained by exposing them to osmium tetroxide vapors, a halo was observed around most of the soil particles (lower left photograph). That this halo has a tridimensional structure

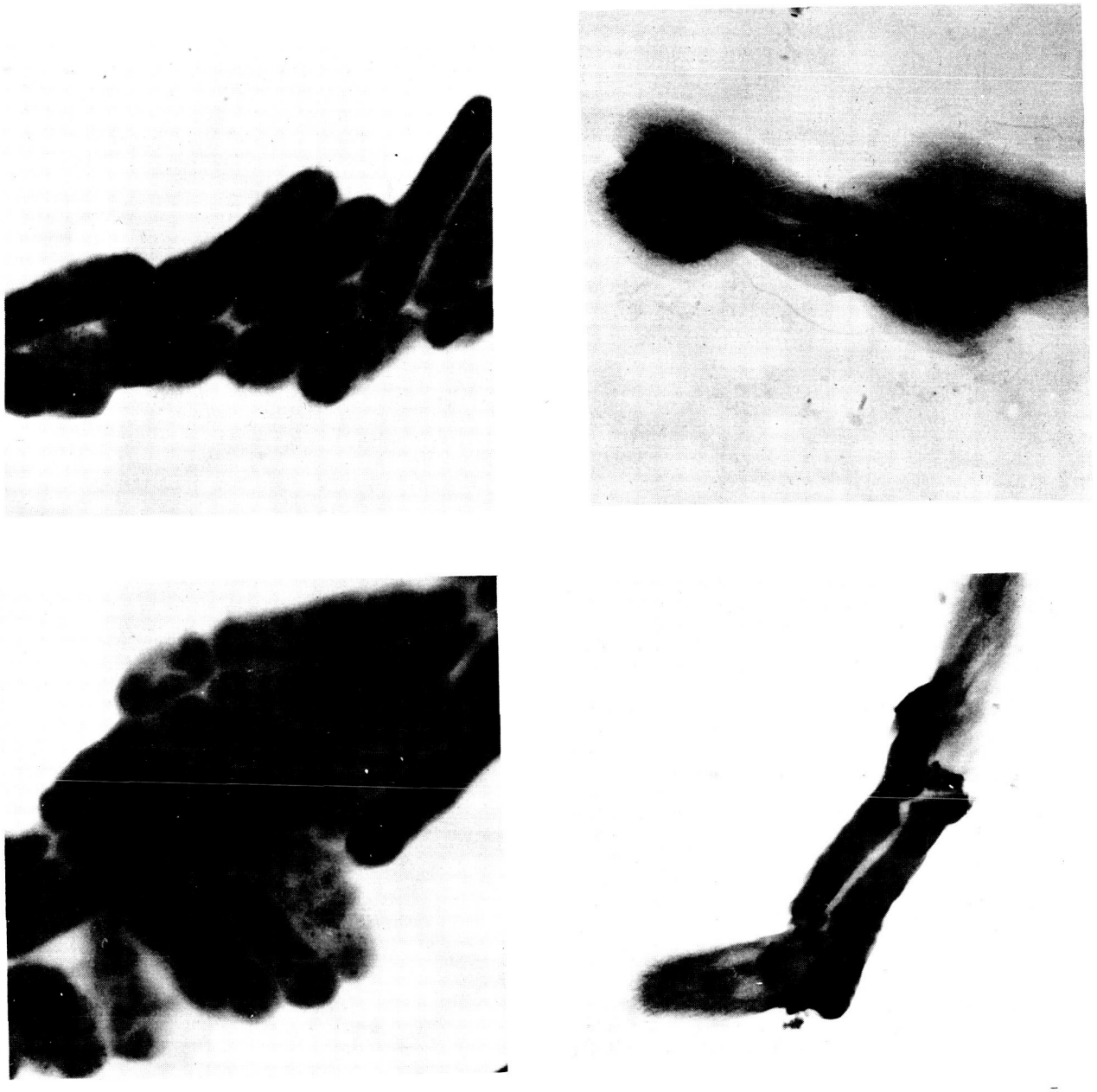


Figure 5. Phosphatase reaction of a derepressed, soil bacterial isolate spread on a carrier film. Incubation in the Gomori medium containing (left) or lacking (right) the substrate.

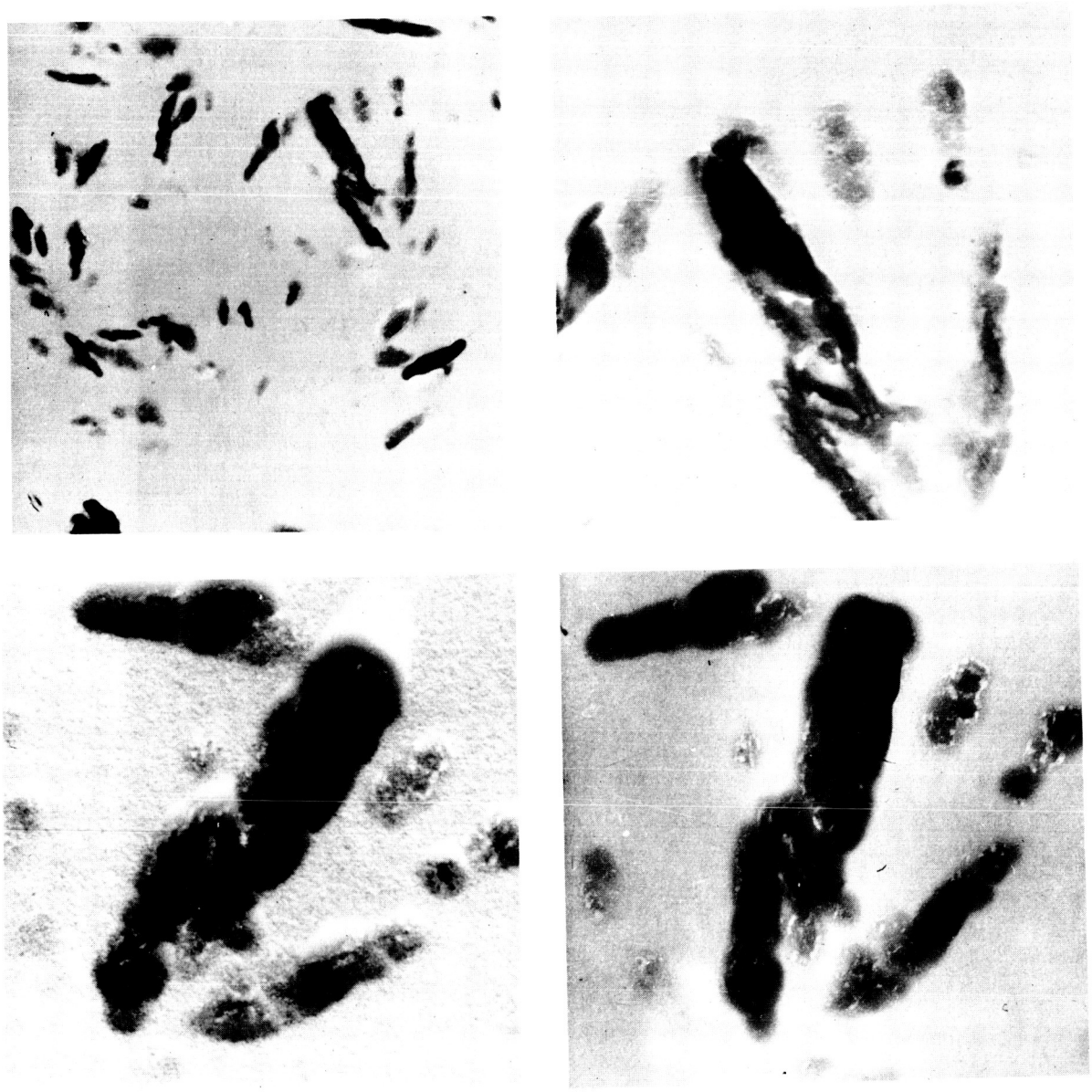


Figure 6. Soil clay embedded in GMA-Epoxy resin mixture. Untreated section (upper). Section stained with OsO_4 (lower left). Section stained and shadowed with uranium (lower right).

that seems to be an uninterrupted coating of the clay particles, is demonstrated in the same section after shadowing with uranium (lower right photograph). Incidentally, sections of clays embedded in either GMA-Epoxy or HPMA resins were not smooth at all, and shadowing always showed that the clay particles were protruding above the surface of the embedding material.

Sectioning of 97% GMA and GMA-Epoxy resin mixture with the diamond knife was found to be very difficult, and the sections usually showed either chattering or folding. This was obviated almost completely by first trimming the resin blocks in such a way that the cutting side of the block facing the knife edge would be at a 30° angle to the vertical axis of the block. Best results were obtained when the diamond knife was set with a clearance angle of 1.5°. HPMA was easier to section, but sections below 400 Å units in thickness were very difficult to see with the aid of reflected diffuse light after floating them on the receiving boat, and thus could not be picked up.

Finally, it should be pointed out that changes either in the concentration of lead salt (below and above the recommended one), or in the kind of lead salt used (15), did not modify in any degree the results in the phosphatase reaction of the materials used.

Discussion

Although in Dublin soil the clay and silt fractions demonstrated a phosphatase activity per unit weight twice as high as in the "coarse" fraction, the contribution of each fraction computed on

a soil composition basis was approximately equal. Part of the phosphatase activity of whole soil (approximately 17%) was unaccounted for and this might be due to some loss of the enzyme during the soil fractionation process.

The highest phosphatase activity per unit weight found in the clay and silt fractions of Dublin soil, agrees with results reported by Haig (7) for soil esterase, and by Hoffmann (9) for carbohydrases and urease in soils. Hochstein (8) found that a soil supernatant fraction showed a phosphatase activity equal to about 10% of the total soil activity, and that essentially all the activity associated with soil could be accounted for in the residual soil fraction. However, his soil supernatant fraction undoubtedly included most of the clay fraction and part of the silt fraction, whereas his residual soil consisted mostly of the so-called "coarse" fraction. The data shown in Table II indicate, on the contrary, that most of the activity associated with whole soil can be accounted for in the clay and silt fractions, and that the pooled supernatant fluids of the latter fractions account for only 1% of the total activity of whole soil.

The data appearing in Table II clearly show, also, that there was no direct relationship between phosphatase activity and detectable bacterial population of the samples examined and agree with the findings presented previously (31). Hoffmann (9) also reported that urease activity, which was found to be higher in the clay fraction than in the silt fraction, was not correlated with microbial numbers.

The fact that silt is low in bacteria, yet is as high in phosphatase activity as clay, argues for the presence of free soil enzymes in the silt fraction.

With regard to the ultrastructural localization of phosphatase activity in soils, no conclusive results were obtained. Although a larger number of electron-dense, crystal-like structures were found in soil clay suspension incubated in the complete Gomori medium than in the corresponding control, and they were mostly associated with the clay particles, this does not constitute valid evidence for the assumption that deposition occurred in the site where the reaction producing these structures took place. The association between these structures and the clay particles could have taken place during the post-incubation washing and sedimentation. The presence of the black, crystal-like structures in the control can be explained as an increased mineralization of the soil clay organic phosphorus stimulated by the optimal conditions provided. In addition to this, the inorganic phosphate already present in the soil clay and presumably capable of reacting with the added lead ions (26) also contributes to the deposition of these black structures.

In the case of soil clay and of clay-phosphatase complexes spread on a carrier film, the lead phosphate formed during the course of the phosphatase reaction probably settles into the incubation medium instead of attaching itself to the site where the phosphate was liberated. It was decided to float the grids carrying the specimens to be tested on the incubation medium rather than to place them in the bottom of extrinsic lead phosphate crystals, but also the

suggestion of a false location of the site of the phosphatase reaction. The inversion of the floating grids did not change the results.

When biological material is used for the histochemical determination of the site of phosphatase activity, diffusion out of the captured reaction products and penetration of contaminating material into either cells or organelles, are prevented in part by the barriers provided by membranes. In an open system, such as in the case of soil particles, diffusion of reaction products and contamination even from distilled water (12) are factors which contribute largely to the complexity of the problem. Furthermore, most biological specimens are not electron-dense and, thus, the localization of captured reaction products is simplified. Soil particles, on the contrary, are very variable in nature and most of them are very electron-dense. Thus, there is no simple way to determine whether the dark areas on the surface of some soil particles are due to the accumulation of lead phosphate or not.

It is interesting to note, also, that bacteria present in the soil clay fraction were not very active hydrolyzers of glycerophosphate, even after a 20-hour incubation period. However, when the phosphatase reaction was carried out on soil bacteria particularly active toward phenolphthalein phosphate and which had been pretreated with a phosphate deficient medium, positive results were then observed in intact bacterial cells incubated in the presence of substrate. The fact that derepressed (phosphate-starved) cells were able to carry out the phosphatase reaction suggests that the procedure was well suited for the purposes.

To avoid the problem of the high electron-density of soil clay particles, ultrathin sections of these particles embedded in water-soluble resins were incubated in the Gomori medium either containing or lacking the substrate. With this procedure a clearcut distinction could not be established between treated samples and the controls. This was also the case for clay-phosphatase complexes embedded in water-soluble resins. It seems that the amount of enzyme left on the rim of the cut particles cannot yield enough reaction products to be detectable after their capture by the lead ions. On the other hand, the resins and the catalysts used to induce their polymerization, as well as the polymerization process in itself, undoubtedly contribute to the inactivation of the enzyme. It should be added that although the Gomori method seems simple to perform, the difficulties involved in getting uniform and reproducible results have been stressed by quite a few of the workers in the field of enzyme histochemistry (1, 2, 25).

In summary, the characteristic complexity of soils in addition to the factor that their enzymatic activities represent very low concentrations (the equivalent of phosphatase activity in one gram of soil is contained in approximately a 10 mg dry weight sample of bacteria), made the handling of this problem extremely difficult. Even in bacteria, where the enzyme system could be said to be concentrated and confined to very small compartments, there has been only one successful attempt to demonstrate the localization of phosphatase activity at the ultrastructural level (3). In this particular case, derepressed cells were used and no electron-dense

materials resembling the captured phosphatase reaction product were found in cells grown in a medium containing inorganic phosphate.

The work reported here on the ultrastructural localization of phosphatase activity does not prove the hyposthesis of the presence of enzymes adsorbed on the soil particles, nor does it deny it. A satisfactory answer to this problem cannot be obtained with the techniques available right now. Perhaps in the near future, with the development of more refined techniques it may become possible to distinguish completely the activity of the soil enzymes per se from other sources of enzyme activity in soils.

Nevertheless, techniques were developed to study soil clay particles either spread on a carrier film or embedded in water-soluble resins. With the floatation technique worked out for the spreading of clay particles on a carrier film, an excellent clay dispersion is obtained. Most of the stack effect, observed with the spraying procedure usually utilized in clay studies, was almost completely eliminated. Thus, there is a better chance of observing any change taking place on the surface of the clays as a result of the accumulation of visible products of a given reaction. The material taken up on the film-covered grid stays there through the whole process of running a reaction and of washing after the reaction is completed. This technique can be also used advantageously for morphological studies of clay in soils, as well as for studies of the interactions between soil particles and microorganisms. In the clay preparation obtained from Oxford Tract soil, for example, kaolinite was easily identified in Figure 3 (lower right photograph) and bentonite was

associated with some other clay particles (lower photographs of Figure 3). The adsorption of small particles of clay on soil bacteria can also be seen in Figure 4.

The sectioning of soil particles embedded in water-soluble resins could help to solve some problems in soil biochemistry and microbiology. Thus, Figure 6 shows the existence of a non-electron dense material surrounding the clay particles. Evidence for this material covering the soil clay particles was not obtained until sections of them were treated with electron-dense stains and shadowed with uranium. Kroth and Page (14) were unable to find evidence that organic matter exists as a capsule around soil particles. However, they made their studies on whole clay particles and did not treat their preparations with electron-dense stains. It is a well-known fact that even the largest organic molecules, such as proteins and nucleic acids, are not electron-dense. In addition to the staining and shadowing of soil particles in sections, there is the possibility of using other treatments for specific purposes, such as the use of hydrofluoric acid solutions to remove the mineral part and to leave the organic portion unchanged.

Summary

After fractionation of Dublin soil, most of the activity associated with whole soil was present in the clay and silt fractions. A small percentage of the activity was contained in the "coarse" fraction. There was no direct relationship between the phosphatase activity and the detectable bacterial population. The fact that

silt is low in bacteria, yet is as high in the phosphatase activity as clay, indicates the presence of free soil enzymes in the silt fraction.

With regard to the ultrastructural localization of phosphatase activity in soils, no conclusive results were obtained with the electron-microscopy techniques. Although a larger number of electron-dense, crystal-like structures (precipitated phosphates) were found in the experimental clay suspensions than in the corresponding control, and they were mostly associated with the clay particles, this was not accepted as a valid evidence for the assumption that deposition occurred in the site where the reaction producing these structures took place. Techniques were developed to study clay particles either spread on a carrier film or embedded in water-soluble resins. With the floatation technique an excellent clay dispersion was obtained. Most of the stack effect, observed with the spraying procedure usually utilized in clay studies, was eliminated. This technique can be used advantageously for morphological studies of clay in soils, as well as for studies of the interactions between soil particles and microorganisms. A method has been developed for sectioning of soil particles embedded in water-soluble resins; it is well suited for applications.

References

1. Barka, T. and Anderson, P. J. Histochemistry. Hoeber, New York-Evanston-London, 1963.
2. Burstone, M. S Enzyme histochemistry and its applications in the study of neoplasms. Academic Press, New York, 1962.
3. Done, J., Shorey, C. D., Loke, J. P. and Pollack, J. K. Biochem. J., 96, 27C (1965).
4. Egaway, T. and Watanabe, Y. Bull, Nat. Inst. Agr. Sci., Ser. B, 14, 173 (1964).
5. Ericson, J. L. E. and Trump, B. F. Histochemie, 4, 470 (1965).
6. Gomori, G. Microscopic histochemistry: principles and practice. University of Chicago Press, Chicago, 1952.
7. Haig, A. D. Some characteristics of esterase and urease-like activity in the soil. Ph.D. Dissertation, Univ. of California, Berkeley, 1955.
8. Hochstein, L. The fluorometric assay of soil enzymes. NASA-CR-50919 (1962).
9. Hoffmann, G. A. PflErnähr. Düng. Bodenk., 85, 97 (1959).
10. Holt, S. J. and Hicks, R. M. J. R. micr. Soc., 81, 173 (1963).
11. Jenny, J. and Grossenbacher, K. Soil Sci. Soc. Amer. Proc., 27, 273 (1963).
12. Kordan, H. A. Science, 149, 1382 (1965).
13. Kotelev, V. V. Microbiology, 29, 659 (1960). (A translation from Mikrobiologiya).
14. Kroth, E. M. and Page, J. B. Soil Sci Soc. Amer. Proc., 11, 27 (1947).

15. Lake, B. D. J. R. micr. Soc., 85, 73 (1966).

16. Leduc, E. H. and Holt, S. J. J. Cell Biol. 26, 137 (1965).

17. Leduc, E., Marinozzi, V. and Bernhard, W. J. R. micr. Soc., 81, 119 (1963).

18. Loeb, T. and Zinder, N. D. Proc. Nat. Acad. Sci., 47, 282 (1961).

19. Luft, J. H. J. biophys. biochem. Cytol., 9, 409 (1961).

20. Malamy, M. H. and Horecker, B. L. Biochem., 3, 1889 (1964).

21. Marinozzi, V. J. J. Ultrastruc. Res., 10, 433 (1964).

22. McGee-Russell, S. M. and Brujin, W. C. Quart. J. micr. Soc., 105, 231 (1964).

23. McLaren, A. D. Soil Sci. Soc. Amer. Proc., 18, 170 (1954).

24. McLaren, A. D. Peterson, G. H. and Barshad, I. Proc. 22, 239 (1958).

25. Pearse, A. G. E. Histochemistry. Theoretical and applied. J. and A. Churchill, Ltd., London, 1960.

26. Poux, N. J. Microscopie, 2, 557 (1963).

27. Sadvir, S. Sci. Tools, 10, 12 (1963).

28. Skujins, J. J., Braal, L. and McLaren, A. D. Enzymologia, 25, 125, (1962).

29. Tooze, J. J. Cell Biol., 26, 209 (1965).

30. Raminirez-Martinez, J. R. and McLaren, A. D. Enzymologia, 30, 243 (1966).

31. Skujins, J. J. and McLaren, A. D. Enzyme Activity in Terrestrial Soil in Relation to Exploration of the Martian Surface. Fourth Semiannual Program Report. NASA-CR-76549 (1966).

B. Biochemistry of Alkane Degradation by Soil Microorganisms.

This report continues the description of the characteristics of four alkane-utilizing bacteria(1). While work was in progress, a study by Baumann of a large number of similar bacteria became available (2). Among the bacteria studied by Baumann were three strains used in our studies: strains HO1-O-22, 3-23, and 4-24. Baumann attempted to characterize his bacteria by their capability of using a large number of compounds as sole carbon and energy source for growth. Therefore, the same tests were applied to the strain WHB-1 isolated by us in order to correlate the characteristics of this strain with Baumann's organisms and an attempt was made to classify strain WHB-1 according to the taxonomic scheme proposed by him.

Materials and Methods

The organisms and techniques used have been described in the previous report (1), except for the following:

Isolation of n-dodecane utilizing organisms. For isolation of n-dodecane utilizing organisms a mineral medium of the following composition was used:

NH_4Cl	1.0/g
K_2HPO_4	2.0g
$MgSO_4 \cdot 7H_2O$	0.2g
$CaCl_2 \cdot 2H_2O$	0.02g
$FeSO_4 \cdot 7H_2O$	0.02g
distilled water	1 liter

Cultures containing this mineral medium and 2% n-dodecane were inoculated with soil and incubated at 30°C on a rotary shaker. Two days later the cultures were streaked on plates containing the mineral medium solidified with 2% agar. The plates were inverted and a few drops n-dodecane were placed in the lid. After two days at room temperature colonies appeared. Organisms were purified on such "mineral-alkane" agar plates, and then streaked on peptone-yeast extract agar plates to give colonies suitable for characterization. In pure cultures the organisms could grow in the mineral medium with n-dodecane as the sole carbon and energy source.

Growth Tests. In preparing the stock solutions for the growth tests, if a compound was insoluble in water, the solution was heated to dissolve it and the sterile stock solutions were prepared while the compound remained dissolved. With some compounds growth did not occur at a substrate concentration of 0.2% but did occur at a lower concentration (as indicated in the Results section).

Tests for aromatic ring fission (3). The organisms were grown in 2.5 x 15 cm tube cultures of 10 ml mineral growth medium containing 0.2% 3,4 - dihydroxybenzoic acid which had been neutralized and sterilized by filtration. After growth the culture was centrifuged the supernatant poured off, and the cells resuspended in 5.0 ml of 0.02 M Tris buffer pH 8.0. The suspension was poured into a 50 ml Erlenmeyer flask, toluenized, and 50 micromoles of 3, 4 - dihydroxybenzoic acid (protocatechuic acid) in 1.0 ml of buffer was added. The flasks were incubated one hour at 30°C without shaking. At the end of this time the color of the suspension was noted and the Rothera

test was carried out as follows. The suspension was centrifuged, the cells were discarded, and the supernatant was saturated with solid ammonium sulfate. A few drops of concentrated NH_4OH and of dilute sodium nitroferricyanide, $Na_2Fe(CN)_5NO \cdot 2H_2O$, solution were added and the solution was mixed and let stand 30 minutes for development of color. The control contained a dilute solution of beta-ketoadipic acid.

Results

Growth tests.

Amino acids tested: L-leucine, D,L-norleucine, L-serine, L-cysteine, L-methionine, L-asparagine, L-glutamine, L-tryptophan, D-tryptophan, L-ornithine, beta-alanine, D,L-alpha-aminobutyric acid, gamma-aminobutyric acid, delta-aminovaleric acid, epsilon-aminocaproic acid, betaine, sarcosine, and creatine.

No strain grew on D,L-norleucine, L-serine, L-cysteine, L-methionine, D-tryptophan, D,L-alpha-aminobutyric acid, delta-aminovaleric acid, epsilon-aminocaproic acid, betaine, sarcosine, or creatine. Strains HO1-O-22, 3-23, and 4-24 could grow on L-leucine, L-asparagine, L-glutamine, L-tryptophan, L-ornithine, beta-alanine, and gamma-aminobutryic acid. Strain WHB-1 could grow only on L-asparagine and L-glutamine. The growth of the strains on L-leucine, L-tryptophan, and L-asparagine was weak.

Other acids tested: oxalic, malanic, suberic, azelaic, maleic, fumaric, D-tartaric, L-tartaric, glycolic, L-malic, citric, itaconic, pyruvic, alpha ketoglutaric, and pantothenic.

No strain grew on oxalic, maleic, D-tartaric, glycolic, itaconic, or pantothenic acid. Only strain 3-23 grew on L-tartaric acid. All four strains grew on fumaric, L-malic, pyruvic, and alpha-ketoglutaric acids. Strains HO1-O-22, 3-23 and 4-24 could grow on citric and malonic acids, and also on suberic and azelaic acids if the concentration of these two acids was 0.1%, but strain WHB-1 could not grow on any of these.

Application of improved culture and detection methods showed that some of previously reported acids (leucine, methionine, tryptophan, suberic, and azelaic acids) could support growth of some of these microorganisms, as described, especially if used in a lower concentration. The previously reported data (1) should be corrected accordingly.

Purines and pyrimidines tested: adenine, guanine, uric acid, uracil, thymine, cytosine.

No strain grew on any of these compounds.

Aromatic compounds tested: benzylamine, phenol, resorcinol, D,L-benzoylalanine; also benzoic, phthalic, meta-hydroxybenzoic, para-hydroxybenzoic, protocatechuic, para-aminobenzoic, phenylacetic, and hippuric acids.

No strains grew on benzylamine, phenol (0.01 - 0.2%), resorcinol, phthalic, meta-hydroxybenzoic, or para-aminobenzoic acids. All strains grew on benzoic (at 0.05%) and protocatechuic acids, and all but strain WHB-1 could grow on D,L-benzoylalanine, para-hydroxybenzoic, phenylacetic, and hippuric acids.

Aromatic ring fission. For all four organisms, no color appeared in the suspension of toluenized cells with protocatechuic acid as substrate after an hour's incubation. When the Rother's test was

applied, a deep purple color, similar to that in the control tube, slowly developed, indicating the presence of beta-ketoadipic acid. It was apparent that the rupture of the aromatic ring was by an ortho cleavage.

Discussion

The organisms studied by Baumann (2) are non-motile, Gram-negative, strictly aerobic, non-dentrifying, coccoid bacteria. They fall into two subgroups: oxidase-positive penicillin-sensitive organisms, and oxidase-negative penicillin-resistant ones. A large proportion of the representatives of the latter group are able to grow on long-chain alkanes as sole carbon and energy source, and in fact, Kallio's strains H01-0-22, 3-23, and 4-24 fall into this group.

Many of the physical and biochemical characteristics of the strain WHB-1, are identical to those of Baumann's oxidase-negative subgroup. An explicit listing of these characteristics will be made when a more detailed comparison has been completed. From the results of the tests completed so far, however, there appear to be no major differences between strain WHB-1 and his oxidase-negative strains.

Baumann assigns his organisms to the genus Moraxella. Although the oxidase-negative strains vary in the pattern of their ability to grow upon organic compounds as sole carbon and energy source, Baumann believes the group as a whole is sufficiently homogeneous to be considered as a single species. He assigns to this group the species name calco-acetica. Therefore, strain WHB-1, previously reported similar to Moraxella lwoffi (1), may be tentatively

classified as being a strain of Moraxella calco-acetica (Baumann).

Summary

The properties of four alkane-metabolizing bacteria were further examined, and in particular, their ability to grow on various organic compounds as sole carbon and energy source. Comparison of these organisms with a group of similar bacteria studied by another laboratory allowed the tentative identification of these bacteria strains of Moraxella calco-acetica (Baumann).

References

1. Skujins, J. J., and McLaren, A. D. Enzyme Activity in Terrestrial Soil in Relation to Exploration of the Martian Surface. Fourth Semiannual Progress Report. NASA-CR-76549 (1966).

2. Baumann, P. Taxonomic and Ecological Studies on the Moraxella Group of Bacteria. PhD Dissertation. University of California, Berkeley. 1966.

3. Stanier, R. Y., Palleroni, N. J., Doudoroff, M. J. Gen. Microbiol., 43, 159 (1966).

C. Adsorption and Reactions of Chitinase and Lysozyme on Chitin.

The currently reported phase of the investigation of the chitinase and lysozyme activity in adsorbed state on chitin is a continuation of the previously described project under the same title (43, 60) on enzyme kinetics in structurally restricted systems.

Materials and Methods

Materials and methods have been described in detail in the previous reports (43, 60). Additional methods are described below.

Dispersed Chitin (43). One single stock preparation of chitin was used throughout (stock: 5.0 mg chitin/ml).

Chitinase was obtained from streptomycete strain 2B. The DEAE-cellulose purified preparations were fractionated through Sephadex G-50 (coarse) column and the first peak of chitinase activity was used for activity and adsorption experiments.

Lysozyme - 2x crystallized (lot 597, Worthington Biochem. Corp., Freehold, N. Y.), dissolved in 0.01 M Na phosphate buffer pH 7.0. Where mentioned, spray dried lysozyme (LYSD 641, same manufacturer) was used.

Absoptivity (extinction coefficient) of chitinase as previously determined: $E_{280}^{mg/ml} = 1.10$; lysozyme: $E_{280}^{mg/ml} = 2.64$ (20, 42)

Adsorption of enzyme protein on chitin was determined by absorptivity measurements of enzyme solutions at 280 mμ before and after addition of chitin following centrifugation.

Chitinase and lysozyme activity was based on the amount at the

released N-acetylglucosamine, i.e., by the DMAB method (43, 61).

It was found that an addition of borate buffer (as used in the DMAB method) to chitin-chitinase or chitin-lysozyme reaction mixtures (1.0 ml buffer to 5.0 ml mixture) raised the pH to 9.3 and stopped the enzymatic reaction. Therefore, to improve the timing of reactions, borate buffer was added directly to the tube of reaction mixture at the end of the incubation time. After mixing and cooling in an ice bath and centrifuging, 1.2 ml of supernatant was withdrawn and used for N-acetylglucosamine detection with the DMAB method. All determinations of adsorption and activity at 0° were carried out in a cold room in order to avoid heating-up of samples during the handling of experimental materials.

Results and Discussion

Chitinase Activity at 0° and $25^{\circ}C$. The experimental data are presented in Figures 7, 8, 9, and 10. The influence of temperature on reaction rates is self-evident. A lag period of 10 to 15 minutes is apparent when a small amount of enzyme is reacting with a large amount of substrate. It is possible that the irregularities in the reaction rates at low enzyme concentrations are caused by the presence of inhibitory substances (for example, Cu^{++} may be adsorbed from the live distilled water used in quantity during the washing procedure of the precipitated chitin).

There exist many reports describing chitinase activity on chitin (6, 14, 18, 27, 29, 34, 35, 38, 39, 47, 49, 51, 53, 57, 58, 61, 62, 63) but only three describe the derivation of the value for Michaelis-

Menten constant for this system.

As we have indicated it before, the Michaelis-Menten equation is not applicable to enzymatic reactions at surfaces, however.

Jeuniaux (3) found the K_m to be 0.010 - 0.011 (g/100 ml) for streptomycete chitinase, using acid precipitated chitin as substrate. A very close value to that was obtained by Unestam (64) for Aphanomyces astaci chitinase with the same type of substrate. Reisert (52) estimated the K_m for Chytriomyces chitinase to be 5.7×10^{-6} M of reducing sugar. C^{14} labelled chitin obtained from Allomyces macrogynus cell walls was used as substrate.

Chitin in its natural state is always associated with other substances, such as protein, $CaCO_3$, various pigments, and waxes (15). The close protein-polysaccharide association in chitinous structures also have been recognized (55). The chitin-protein linkages in cockroach mucoprotein has been studied by Lipke and Graves (37). Chitin may occur in α-, β-, or γ-forms (54). The rates of which these forms of chitin are hydrolyzed by chitinases are different. Because of its crystallinity α-chitin is the least readily attacked by enzymes (23, 24).

The need for a natural, nondegraded chitin with unaltered chain lengths for the use as substrate has been indicated by Hackman and Goldberg (23). The authors describe the preparation of two types of chitins from cuttlefish shell: one in a stable, dispersed state (α-form) and the other in powdered (γ-) form. These chitins are considered to be in "natural" form as no strong acids or alkalies are

used in their preparation. The presence of β-type in the cuttlefish chitin has been confirmed by x-ray and infra-red spectral studies (45). Hackman and Goldberg (24) have further investigated the chitins from different animal sources using enzymic digestion, acidic hydrolysis, deuterium exchange, infra-red spectral adsorption, and differential thermal analysis. They have shown that chitins have different structures affecting the accessibility of reagents to the chains and the extent of digestion. Not only do the structures of α- and β- chitins differ but the differences occur also within the α- and the β-chitins. Only about 30% of the total structure of α-chitin is in an organized, crystalline form. β-chitins prepared from cuttlefish shell and squid pen contain significant amounts of free amino groups. The free amino groups are in less symmetrically organized parts of the structure.

The use of powdered chitins has shown that the rate of hydrolysis is very much dependent on particle size (23), as expected. Even if apparently homogenous chitin from an animal source is isolated (45), there remain considerable difficulties to prepare it in a form where all the particles are of the same size. In acid precipitated chitins the size and form varies considerably, even after repeated sedimentation attempts. An attempt to digest the small particles with chitinase did not succeed as all particles were diminished in size at the same rate. Also, it is not known yet at what conditions, especially with respect to the pH, the ionic composition, and the ionic strength of solutions a complete desorption of chitinase from chitin takes place.

The use of carboxymethyl chitin (28) or hydroxyethyl (glycol) chitin (27, 47) is convenient because these substances are soluble. These substrates, however, do not represent the reactions in nature on natural chitins. A promising method is the use of C^{14}-labeled chitin (52) obtained from cell walls of Allomyces macrogynus (1, 48).

Chitinase has been obtained from many plant and animal sources (17, 30, 46, 62), but it has not been crystallized yet. There exist considerable differences among chitinases with respect to optimum pH and temperature (30, 46, 52). Examination of the activities of different fractions of chitinase from the same source obtained by chromatography (29, 39, 47, 50) have led to a conclusion that chitin may be degraded in sequence by more than one enzyme. If the reaction is followed by turbidity methods, the apparent decrease of the substrate is faster than in the case when the same reaction is followed by the analysis of the released end products. The decrease in turbidity could take place without a release of free N-acetylglucosamine (47).

These inherent difficulties encountered in estimating K_m for chitinases explain why only a few such attempts have been made. A more complete evaluation of the obtained data will be possible only after we gain some more detailed experimental information regarding the contribution to the activity measurements by the various types of chitin and chitinases which might be present in our system.

Behavior of Heat-denatured Chitinase. Chitinase solutions were heated in a water bath at 56° and 70°C. Aliquots were taken

at different time intervals and the ability to adsorb on chitin at 0° and 25°C, as well as the activity (at 37°C) were compared. The data are presented in Figures 11 and 12.

The activity of chitinase heated at 56° was decreased by about 88% after one hour and by 97% after 3 hours. Similarly, the ability to adsorb on chitin was also decreased. Chitinase exposed to 70°C lost all of its activity in about 5 minutes. It should be noted that the absorbance method was used for the estimation of the amount of protein present can assure only about ±5 μg/ml precision. A sufficient number of experiments have been performed with chitinase exposed to 70°C for 30 minutes, where the loss of the ability to adsorb on chitin was complete.

Highly active chitinase (29, 38) has been obtained by adsorption on chitin at pH 5.2 and pH 6.8, respectively; such method of purification facilitated the separation of active and inactive enzyme proteins.

Unestam (63) reports that Aphanomyces chitinase is strongly adsorbed on chitin and that it is better protected against the effects of heating while adsorbed. This is in agreement with Tracey's (62) observations that chitinase of Lycoperdon resists the effects of storage better at 37° if chitin is present.

Benjaminson et al. (5) observed that ferritin-labeled chitinase had "tied up" a large percentage of combining sites on chitin. The use of ferritin-labeled chitinase opens a way for electron microscopy in studies of its adsorption on chitin.

Titration of Chitinase. Active and heat-denatured chitinase solutions were titrated in order to observe eventual differences between their

respective titration curves. The titration curve of active chitinase is shown in Figure 13. The titration curve of the heat-denatured chitinase showed a possible shift of about 0.04 pH unit to the acid side. However, such a slight shift requires further examination and statistical evaluation. Further evaluation of titration curves could provide information concerning the chitinase molecule, its pK_a , and amino and carboxyl groups. A crude chitinolytic enzyme system from Chytriomyces sp. has been titrated by Reisert (52).

Lysozyme activity on Chitin at 0° and $25^{\circ}C$. The experimental results are shown in Table III. The reaction rate at 0° based on the release of N-acetylglucosamine was very slow and close to the sensitivity of the method. The depolymerization was more evident, based on the increase in transmittancy.

At 25° there was a measurable rate of N-acetylglucosamine release. Spray dried ensozyme was used for these experiments.

Desorption of lysozyme. Crystallized lysozyme was used for experiments described below.

While repeated experiments with chitinase did not show any signs of desorption (Fig. 18) lysozyme was desorbed by a fast and considerable rate, as shown in Figures 14, 15, and 16. Desorption is temperature dependent, as expected, and the rate is faster at higher temperatures.

The experimental evidence has shown that the adsorption of lysozyme and of chitinase on chitin takes place within seconds and it is not measurable with our presently used methods. Desorption of lysozyme is also very rapid.

Table III

LYSOZYME ACTIVITY ON CHITIN AT 0° and 25°C

Chitin: 1.0 mg. per ml.
pH 5.5, 0.05 M Na phosphate-acetate buffer
Total volume in each tube: 5.0 ml

Temperature: 0°

Time	Enzyme Protein µg/ml			
	413	414	838	845
	N-acetylglucosamine released - µg/ml			
1 min.	---	2.2	---	3.0
2 min.	---	2.2	---	3.0
3 min.	---	2.2	---	3.0
5 min.	2.2	2.5	3.0	3.0
10 min.	2.2	3.0	3.0	3.8
20 min.	2.5	3.0	3.8	3.8
30 min.	3.0	3.8	4.4	4.4
1 hr.	4.1	4.1	5.7	5.7
2 hrs.	4.1	4.4	7.0	6.3
4 hrs.	4.1	---	7.0	---
E:S=1:—>	2.42	2.41	1.19	1.18
1/S =	0.413	0.414	0.838	0.845

Temperature 25°

Time	Enzyme Protein µg/ml							
	324	402	524	648	811	992	1165	1777
	N-acetylglucosamine released - µg/ml							
1 min.	0.0	0.3	0.6	0.3	1.3	1.3	1.9	2.5
2 min.	0.3	0.6	1.0	0.6	1.0	1.9	2.5	3.0
3 min.	1.0	1.0	2.5	1.3	1.9	3.0	3.0	4.5
5 min.	1.9	1.3	3.0	2.2	2.5	3.8	4.5	5.5
10 min.	3.0	3.0	4.5	4.5	4.5	5.4	6.5	7.5
20 min.	5.0	5.0	6.3	6.5	7.0	7.3	8.2	9.5
30 min.	6.4	7.5	7.3	7.5	8.5	9.0	10.8	10.8
1 hr.	9.0	8.5	10.1	10.8	11.5	13.4	14.0	14.5
2 hrs.	12.0	12.0	14.6	15.0	---	19.7	19.0	21.0
3 hrs.	14.5	16.5	19.1	19.5	---	24.2	23.5	25.5
E:S=1:—>	3.09	2.49	1.91	1.54	1.23	1.01	0.86	0.565
1/S=	0.324	0.402	0.524	0.648	0.811	0.992	1.165	1.777

Adsorption and Activity of heat-denatured lysozyme. Spray dried lysozyme was dissolved in 0.01 M Na phosphate buffer, pH 7.0, 1.0 mg per ml. The enzyme was inactivated by keeping the solution in a boiling water bath for 10 hours (41). The slight amount of precipitate formed was removed by high speed centrifugation.

The denatured lysozyme was completely inactive towards chitin and was not adsorbed (Figure 17). Experiments with partly denatured lysozyme are in progress.

Pertinent information. Despite of the fact that chitin is not the main substrate for lysozyme much more is known about adsorption of lysozyme on chitin than about adsorption of chitinase on its substrate.

Nozu (44) suggested the use of chitin as a specific adsorbent for lysozyme that does not adsorb other proteins. However, a recent report shows that β-fructofuranosidase also is firmly adsorbed on it (66). Following Nozu's suggestion Cherkasov and Kravchenko (13) succeeded in obtaining pure lysozyme by subjecting raw extract to repeated adsorption and desorption cycles on chitin and continued the studies of the behavior of lysozyme on a chitin containing chromatography column (12).

The binding of N-acetylglucosamine and 2 other inhibitors specifically to the same site on lysozyme molecule was shown by x-ray analysis (31), following the observation that the presence of N-acetylglucosamine is inhibiting the lysozyme activity on chitin (Wenzel et al., 65) X-ray crystallographic (8, 31) physical (26) and chemical studies (25, 56) have shown that tryptophan residues

are present at the substrate binding sites of lysozyme.

The binding of chitin oligosaccharides to lysozyme has been investigated by Dahlquist et al. (16) by spectral analysis of lysozyme in the presence of these compounds. Lehrer and Fasman (36) obtained fluorescence data of lysozyme-substrate complexes showing that tryptophan and carboxyl groups are involved in the binding region. Shinitzky et al. (59) investigated the influence of inhibitory sugars on fluorescence of lysozyme. Glazer and Simmons examined the structure-function relationship in lysozyme by applying the methods of circular dichroism spectral analysis (7).

The importance of disulphide bonds for the activity of lysozyme has been studied by Fraenkel-Conrat et al. (19) and by Goldberger and Epstein (22). The cleavage of disulphide bonds was accomplished with the aid of thioglycolate or mercaptoethanol in urea, or with the use of sulphite (1, 2, 4, 11). While the chemical structure of lysozyme molecule was established by Jolles et al. (32, 33) and by Canfield (9, 10), the three-dimensional structure was worked out by Phillips and his group (8, 31).

Summary

Data regarding adsorption, enzymatic kinetics and activity of a streptomycete chitinase and of egg-white lysozyme on chitin at 0° and 25° are presented. With chitin as a substrate a desorption of lysozyme was observed whereas chitinase did not exhibit this phenomenon. Heat-denaturation of both enzymes resulted not only in a complete loss of activity but the proteins also lost the ability to adsorb on chitin.

References

1. Aronson, J. M. Ph.D. thesis, University of California, Berkeley (1958).
2. Azari, P. Arch. Biochem. Biophys. 115, 230 (1966).
3. Azari, P. R. and R. E. Feeney. Arch. Biochem. Biophys., 92, 44 (1961).
4. Bailey, J. L. and R. D. Cole. J. Biol. Chem. 234, 1733 (1959).
5. Benjaminson, M. A., S. L. Basil, F. J. D'Oria, and S. L. Carito. Nature, 211, 1275(1966).
6. Berger, L. R. and D. M. Reynolds. Biochim. Biophys. Acta, 29, 522 (1958).
7. Beychok, S. Science, 154, 1288 (1966).
8. Blake, C. C. F., D. F. Koenig, G. A. Mair, A.C.T. North, D. C. Phillips, and V. R. Sarma. Nature, 206, 757 (1965).
9. Canfield, R. E. J. Biol. Chem., 238, 2698 (1963).
10. Canfield, R. E. and A. K. Liu. Ibid., 240, 1997 (1965).
11. Cecil, R. and G. R. Wake. Biochem. J., 82, 401 (1962).
12. Cherkasov, I. A., Kravehenko, N. A. and Kaverzheva, E. D. Doklady Akad. Nauk SSSR, 170, 213 (1966).
13. Cherkasov, I. A. and N. A. Krauchenko. Izobret., Prom. Obraztsy, Tovarnye Znaki, 43, 8 (1966).
14. Clans, L. Arch. Mikrobiol., 40, 17 (1961).
15. Conrad, J. Science and Technology, Vol. 3, 695 (1965).
16. Dahlquist, F. W., L. Jao and F. Raftery. Proc. Natl. Acad. Sci. U. S., 56, 26 (1966).
17. Dajoz, R. Sci. Progr. Nat. (Paris), No. 3664, 317 (1965).

18. Elorza, M. V., E. M. Ruiz, and J. R. Villanueva. Nature, 210, 442 (1966).

19. Fraenkel-Conrat, H., E. D. Mohammad, E. D. Ducay, and D. K. Mechan. J. Am. Chem. Soc., 73, 625 (1951).

20. Fromageot, C. and G. Schnek. Biochim. Biophys. Acta, 6, 113 (1950).

21. Glazer, A. N. and N. S. Simmons. J. Am. Chem. Soc., 88, 2335 (1966).

22. Goldberger, R. F. and C. J. Epstein. J. Biol. Chem., 238, 2988 (1963).

23. Hackman, R. H. and M. Goldberg. Analytical Biochem., 8, 397 (1964).

24. Hackman, R. H. and M. Goldberg. Australian J. Biol. Sci., 18, 935 (1965).

25. Hartdegen, F. J. and J. A. Rupley. Biochim. Biophys. Acta, 92, 625 (1964).

26. Hayashi, K., T. Imoto, and M. Funatsu. Ibid., 54, 381 (1963).

27. Hayashi, K., N. Yamasaki, and M. Funatsu. Agr. Biol. Chem. (Tokyo), 28, 517 (1964).

28. Hultin, E. Acta Chem. Scand., 9, 192 (1955).

29. Jeuniaux, C. Arch. Intern. Physiol. et Biochim., 64, 522 (1956) Ibid, 67, 597 (1956).

30. Jeuniaux, C. "Chitine et Chytinolyse", Masson et C^{ie}, Éditeurs, Paris VI^{e}, 1963.

31. Johnson, L. N., D. C. Phillips. Nature, 206, 761 (1965).

32. Jollès, J., Jauregui-Adell, J., and Jollès, P. Biochim. Biophys. Acta, 78, 68 (1963).

33. Jollès, P., Jauregui-Adell, J. and Jollès J., C. R. Head. Sci. Paris 258, 3926 (1964).

34. Kawasaki, C., Y. Ito, and G. Kurata. Hakko Kogaku Zasshi, 42, 212 (1964).

35. Kojima, J. and Y. Koase. Meiji Seika Kenkyu Nempo, 12 (1965).

36. Lehrer, S. S. and G. D. Fasman. Biochem. Biophys. Res. Commun., 23, 133 (1966).

37. Lipke, H. and B. Graves. Federation Proceedings, 25, (2 PT. 1), 706 (1966).

38. Lloyd, A. B., R. L. Neveroske, and J. L. Lockwood. Phytopathology, 55, 871 (1965).

39. Lunt, M. R. and P. W. Kent. Biochim. Biophys. Acta, 44, 371 (1960).

40. Maksimow, V. I., E. D. Kaverzneva, and N. A. Kravechenko. Biokhimiya, 30, 1007 (1965).

41. McLaren, A. D. Soil Sci. Soc. Amer. Proc., 18, 170 (1954).

42. McLaren, A. D. J. Physical Chemistry, 58, 129 (1954).

43. McLaren, A. D. and Skujins, J. J. Third Semiannual Progress Report, Space Sciences Laboratory, University of California, Berkeley, January 14, 1966; NASA-CR-70058.

44. Nozu, H. Osaka Daigaku Igaku Zasshi, 12, 1531 (1960).

45. Okafor, N. Soil Sci., 101, 193 (1965).

46. Okutani, K. Bulletin of the Misaki Marine Biological Institute, Kyoto University, No. 10, 1 (1966).

47. Otakara, A. Agr. Biol. Chem. (Tokyo) 25, 50 (1961), 25, 54 (1961), 25, 494 (1961), 26, 30 (1962), 27, 454 (1963), 28, 745 (1964), 28, 811 (1964).

48. Poter, C. A. and E. G. Jaworski. Biochemistry, 5, 1149 (1966).

49. Powning, R. F. and H. Irzykiewicz. Nature, 200, 1128 (1963).

50. Powning, R. F. and H. Irzykiewicz. Comp. Biochem. Physiol., 14, 127 (1965).

51. Prauser, H., and W. Koehler. Z. Allgem. Mikrobiol., 5, 308 (1965).

52. Reisert, P. S. Diss. Abstr., 26, (6), 3003 (1965).

53. Reynolds, D. M. J. Gen. Microbiol., 11, 150 (1954).

54. Rudall, K. M. Advan. Insect. Physiol., I, 257 (1963).

55. Rudall, K. M. Struct. Function Connect. Skeletal Tissue, Proc., St. Andrews, Scot. 1964, 191 (Pub. 1965).

56. Rupley, J. A. Science, 150, 382 (1965).

57. Salton, M. R. J. and J. M. Ghuysen. Biochim. Biophys. Acta, 36, 552 (1959).

58. Salton, M. R. J. and J. M. Chuysen. Biochim. Biophys. Acta, 45, 355 (1960).

59. Shinitzky, M., V. Grizaro, D. M. Chipman and N. Sharon. Arch. Biochim. Biophys., 115, 232 (1966).

60. Skujins, J. J. and McLaren, A. D. Fourth Semiannual Progress Report, Space Sciences Laboratory, University of California, Berkeley, July 12, 1966; NASA-CR-76549.

61. Skujins, J. J., H. J. Potgieter, and M. Alexander. Arch. Biochem. Biophys., 111, 358 (1965).

62. Tracey, M. V. Biochem. J., 61, 579 (1955).

63. Unestam, T. Physiol. Plant., 19, 15 (1966).

64. Unestam, T. Personal communication.

65. Wenzel, M., H. P. Lenk and E. Schütte. Z. Physiol. Chem., 327, 13(1962).

66. Yabuki, M. Nippon Nogei Kagaku Kaishi, 40, 201 (1966).

FIGURE 7

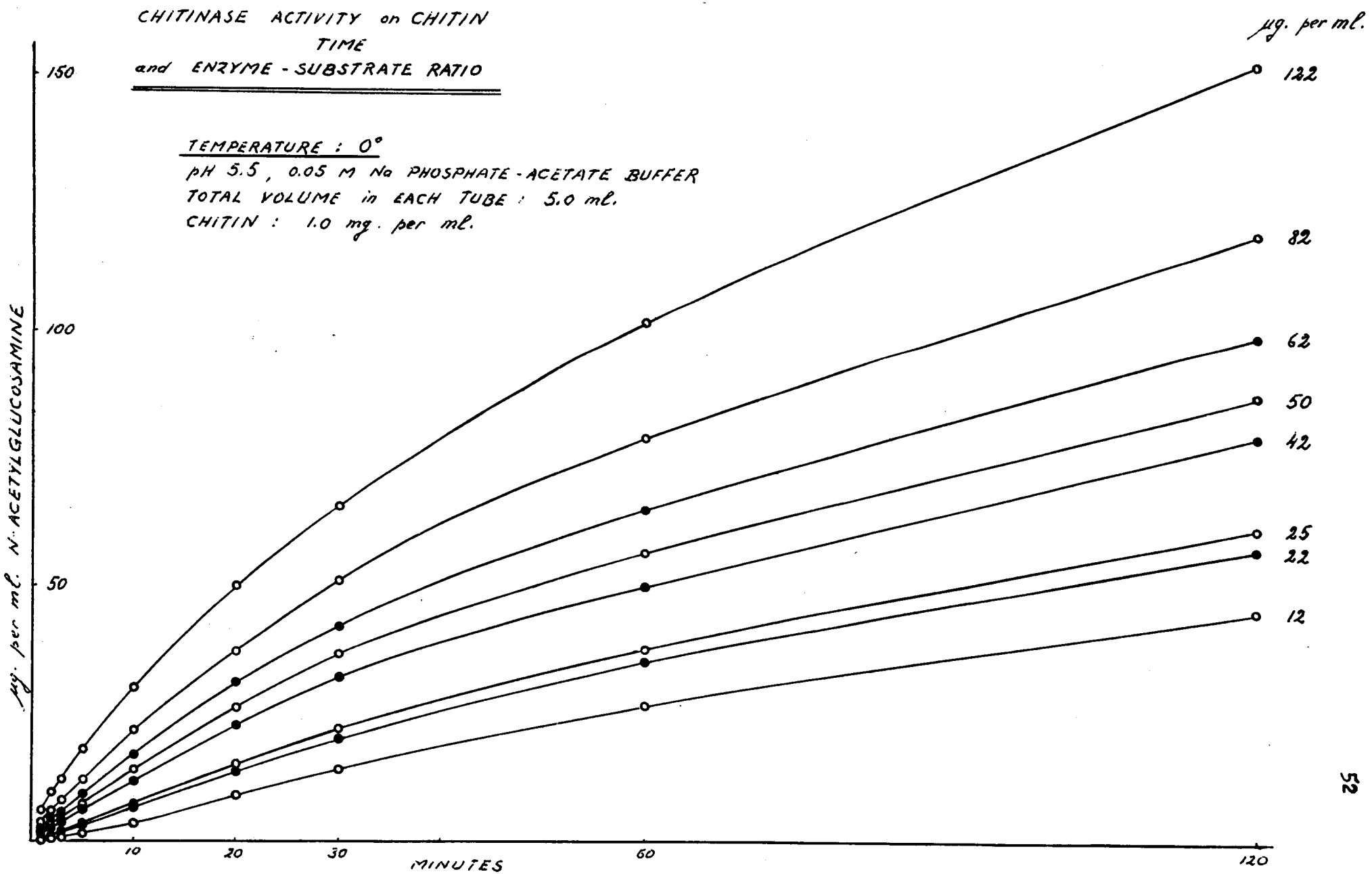

CHITINASE ACTIVITY on CHITIN
TIME
and ENZYME - SUBSTRATE RATIO
TEMPERATURE : 0°
pH 5.5 , 0.05 M Na PHOSPHATE - ACETATE BUFFER
TOTAL VOLUME in EACH TUBE : 5.0 ml.
CHITIN : 1.0 mg. per ml.
µg. per ml.
122
82
62
50
42
25
22
12
µg. per ml. N-ACETYLGLUCOSAMINE
150
100
50
10
20
30
60
120
MINUTES

FIGURE 8

CHITINASE ACTIVITY on CHITIN
ENZYME-SUBSTRATE RATIO
and TIME

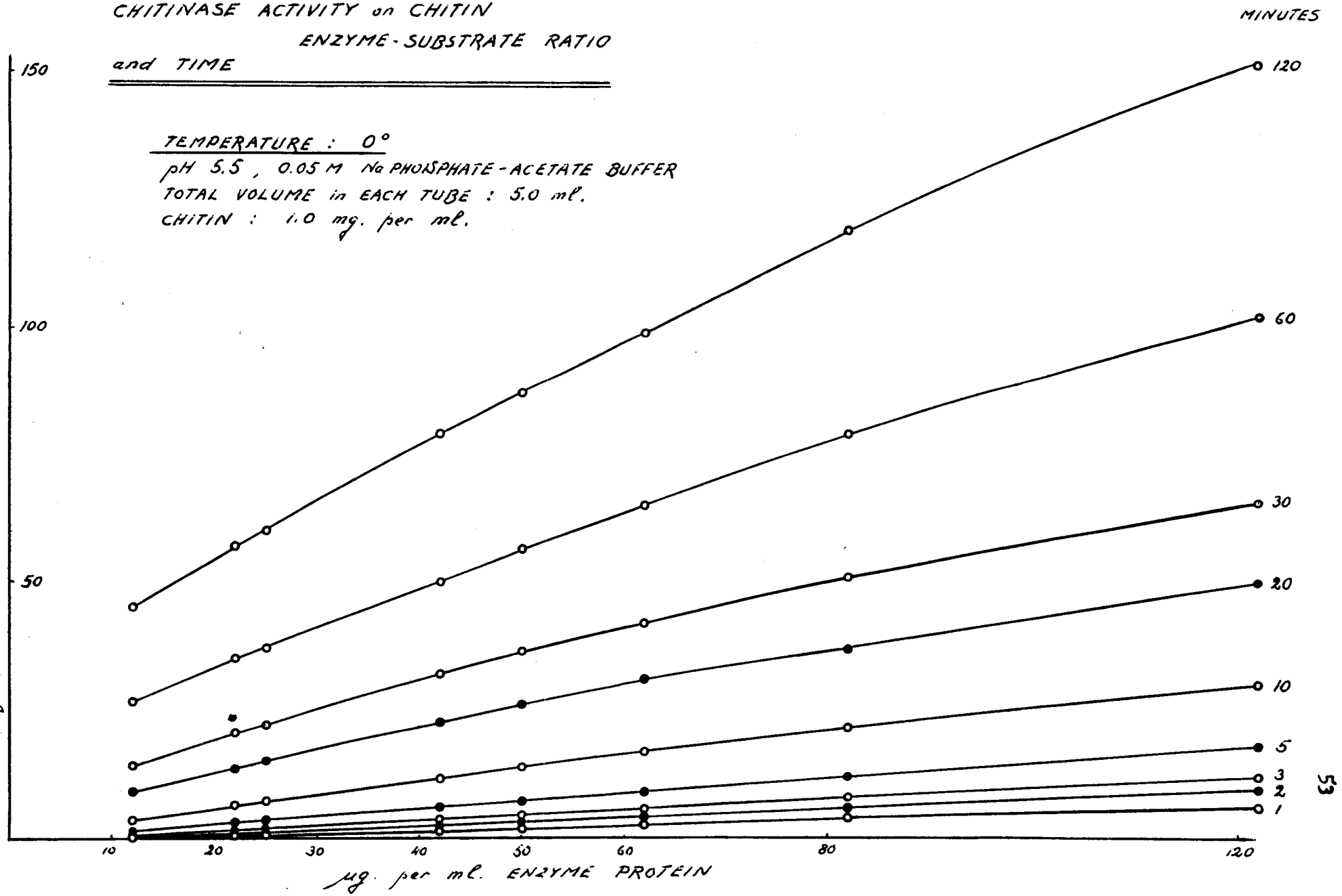
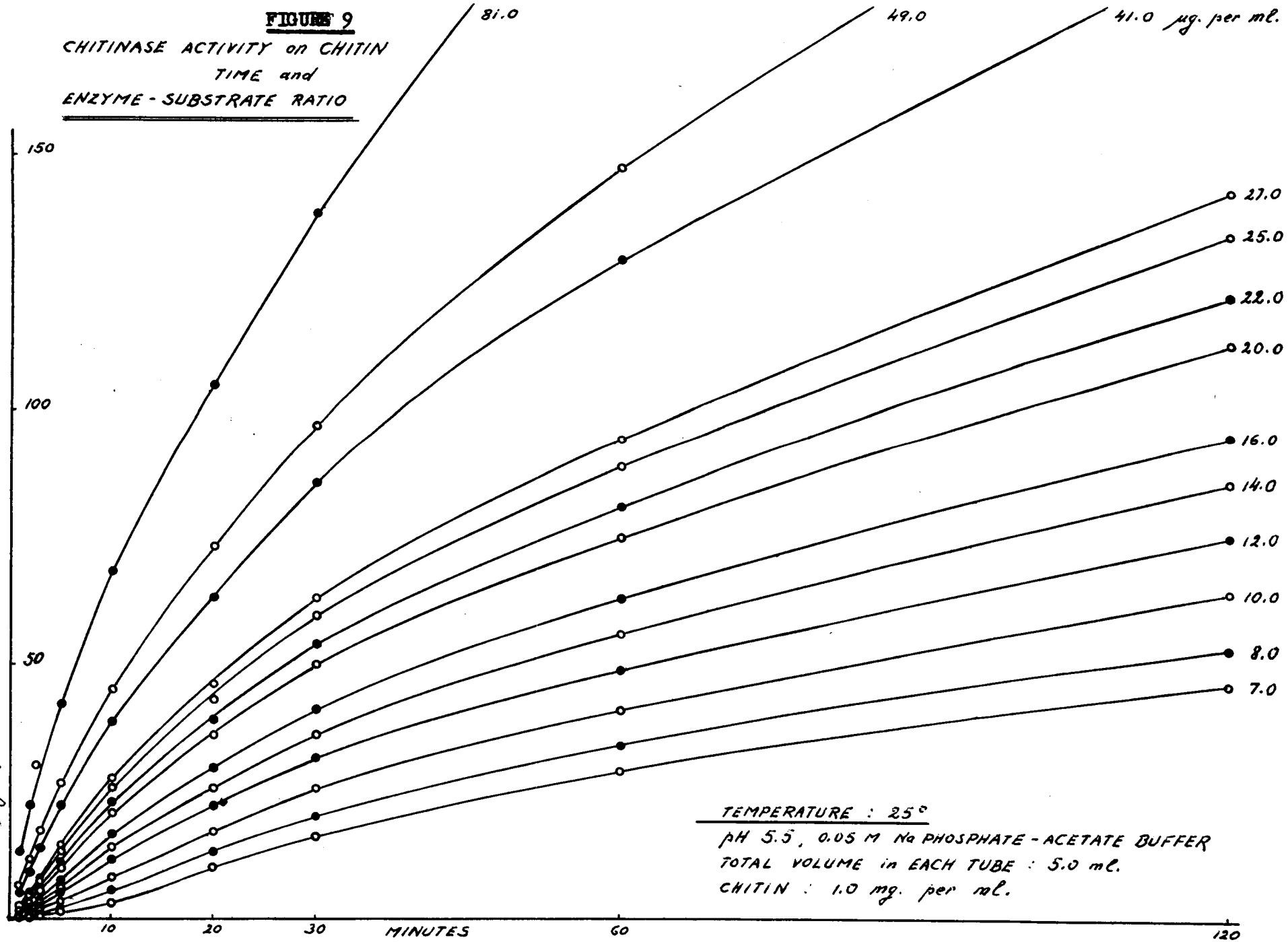


FIGURE 9

CHITINASE ACTIVITY on CHITIN
TIME and
ENZYME - SUBSTRATE RATIO



TEMPERATURE : 25°
pH 5.5, 0.05 M Na PHOSPHATE - ACETATE BUFFER
TOTAL VOLUME in EACH TUBE : 5.0 ml.
CHITIN : 1.0 mg. per ml.

FIGURE 10

CHITINASE ACTIVITY on CHITIN
ENZYME-SUBSTRATE RATIO
and TIME

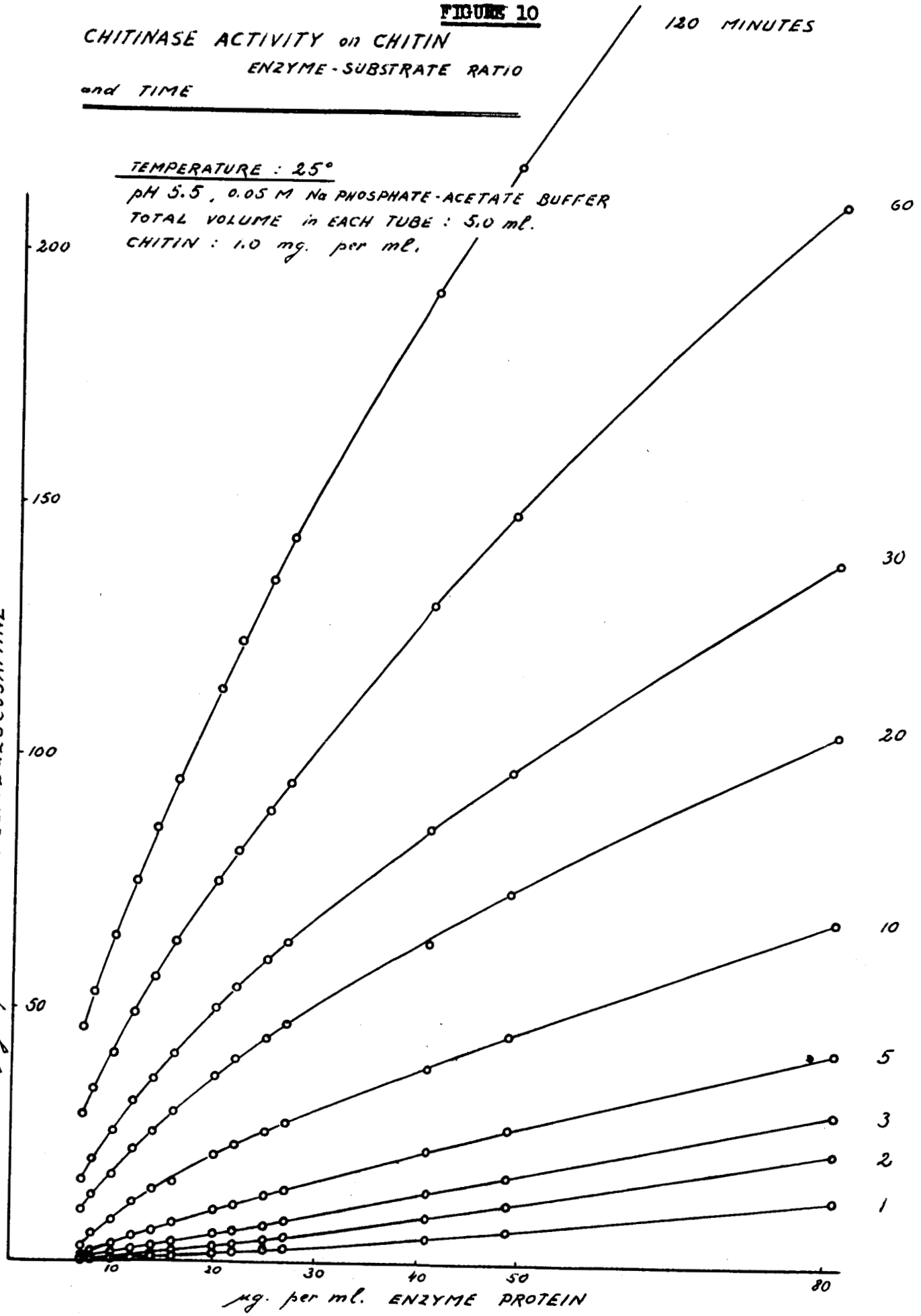
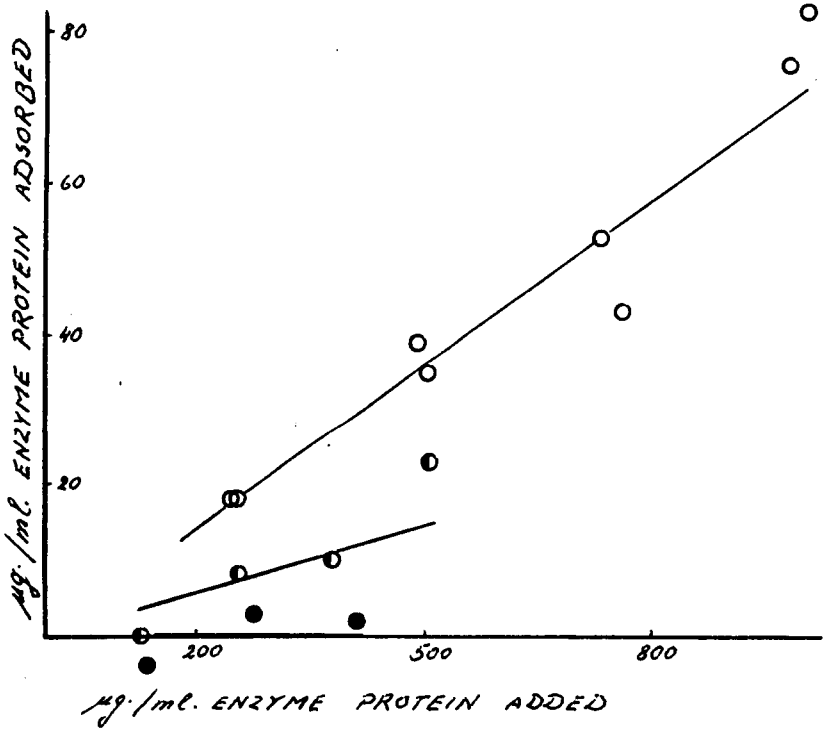


FIGURE 11

ADSORPTION of ACTIVE and HEAT DENATURED CHITINASE on CHITIN



TEMPERATURE : 0°

CHITIN : 50 μg. per ml.

pH 5.5, 0.05 M Na PHOSPHATE-ACETATE BUFFER

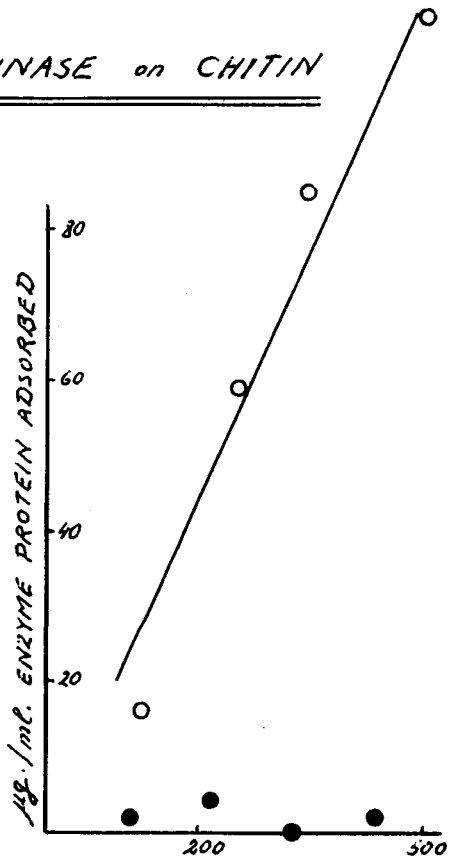
TOTAL VOLUME in EACH TUBE : 5.0 ml

REACTION (ADSORPTION) TIME : 1 MINUTE

CHITINASE: ○ ACTIVE ENZYME

◐ PARTLY HEAT DENATURED ENZYME (2 minutes at 70° - circa 3% original activity)

● COMPLETELY HEAT DENATURED ENZYME (30 minutes at 70° - NO ACTIVITY)



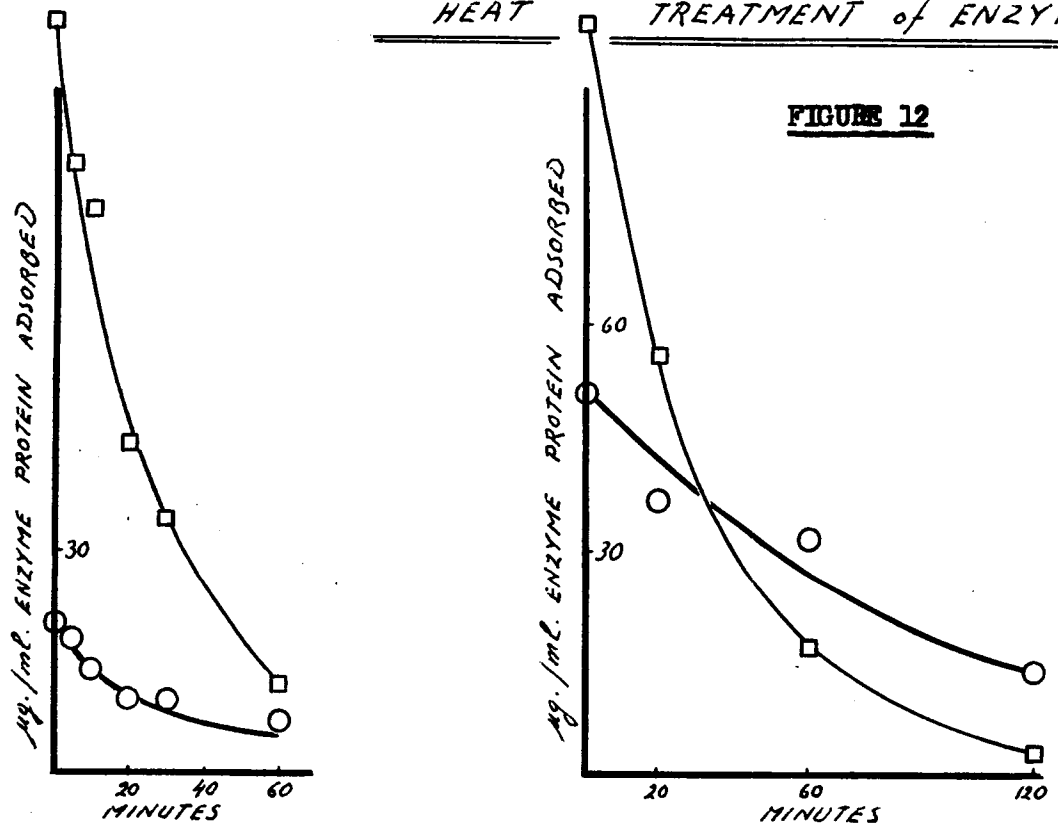
TEMPERATURE : 25°

CHITIN : 500 μg. per ml.

LOSS of ACTIVITY and ADSORPTION of CHITINASE on CHITIN

HEAT TREATMENT of ENZYME

FIGURE 12



TIME - CHITINASE TREATED at 56° BEFORE ADSORPTION

CHITINASE : 138 µg. per ml.

CHITIN : 500 µg. per ml.

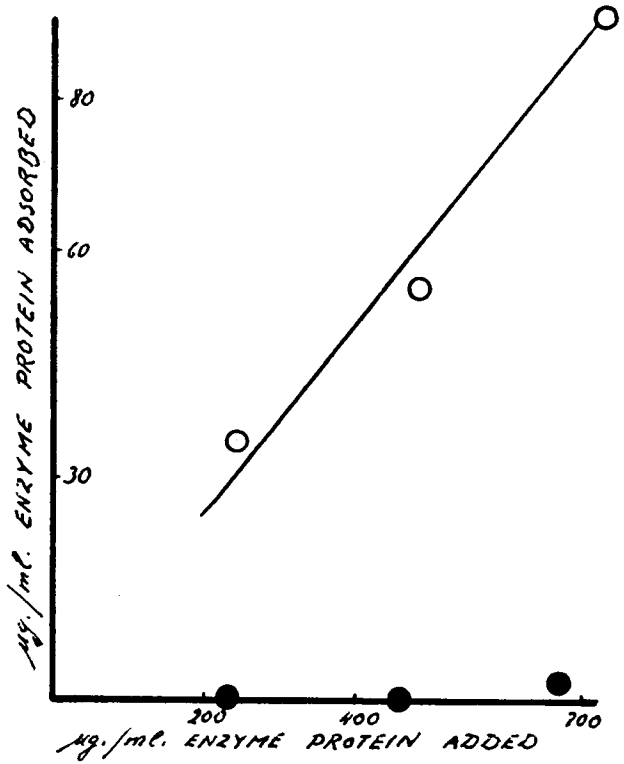
pH 5.5, 0.05 M Na PHOSPHATE-ACETATE BUFFER

TOTAL VOLUME in EACH TUBE : 5.0 ml.

ADSORPTION TIME : 1 MINUTE

TEMPERATURE : 25°

□—□ RELATIVE ACTIVITY, in %



CHITIN : 500 µg. per ml

CHITINASE : 540 µg. per ml.

RELATIVE ACTIVITY - 100 %

● HEATED at 70° for 30 MINUTES

ACTIVITY - NIL

pH 5.5, 0.05 M Na PHOSPHATE-ACETATE BUFFER

TOTAL VOLUME in EACH TUBE - 5.0 ml.

ADSORPTION TIME : 1 MINUTE

TEMPERATURE : 25°

FIGURE 13

TITRATION CURVE of STREPTOMYCETE CHITINASE

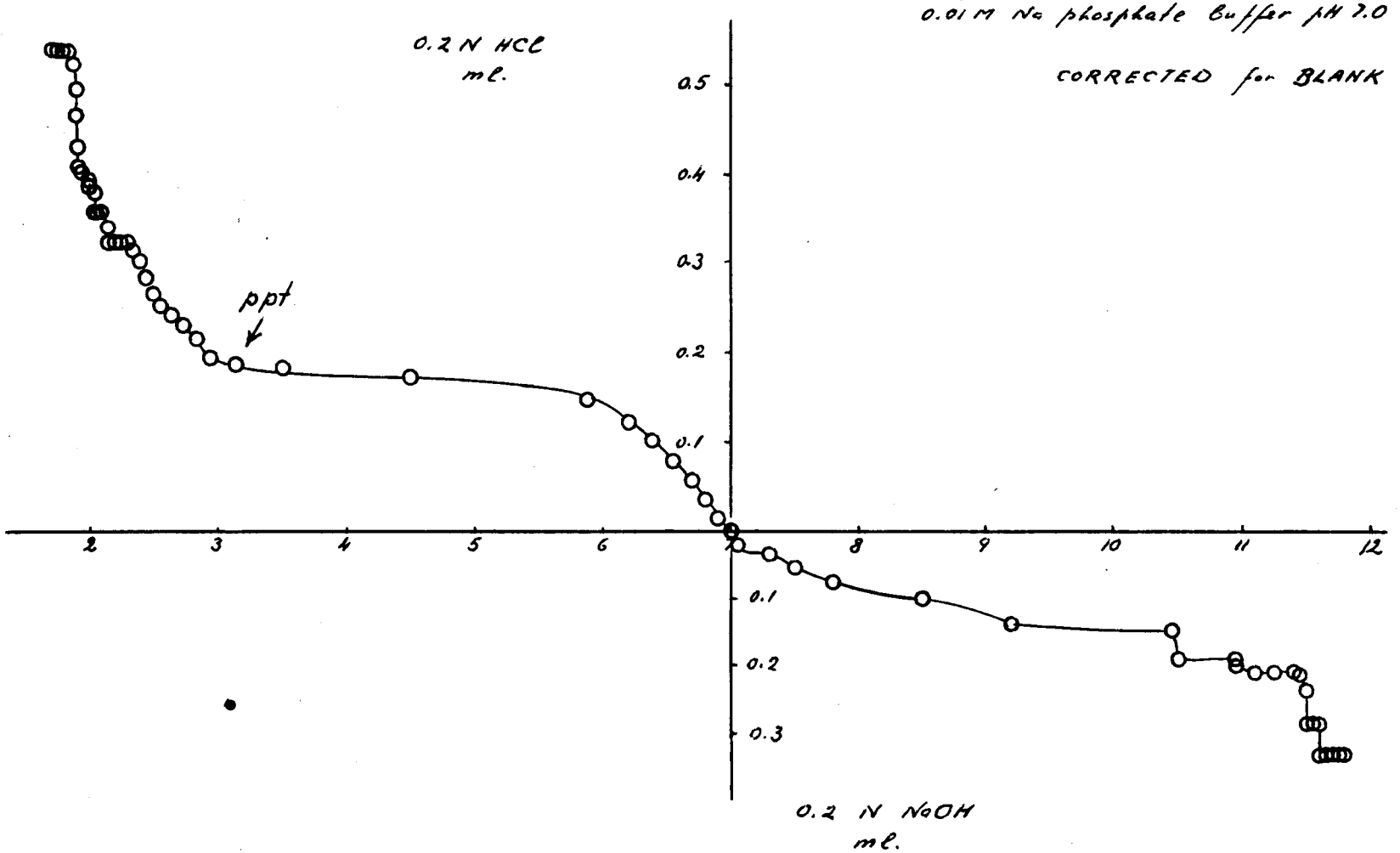


FIGURE 14

LYSOZYME ADSORPTION and ACTIVITY on CHITIN

TEMPERATURE : 0°

pH 5.5, 0.05 M Na PHOSPHATE-ACETATE BUFFER

TOTAL VOLUME in EACH TUBE : 5.0 ml.

CHITIN : 1.0 mg. per ml.

LYSOZYME : 838 µg. per ml.

○ ENZYME PROTEIN ADSORBED

○ N-ACETYLGLUCOSAMINE RELEASED

413 µg. per ml.

● ENZYME PROTEIN ADSORBED

● N-ACETYLGLUCOSAMINE RELEASED

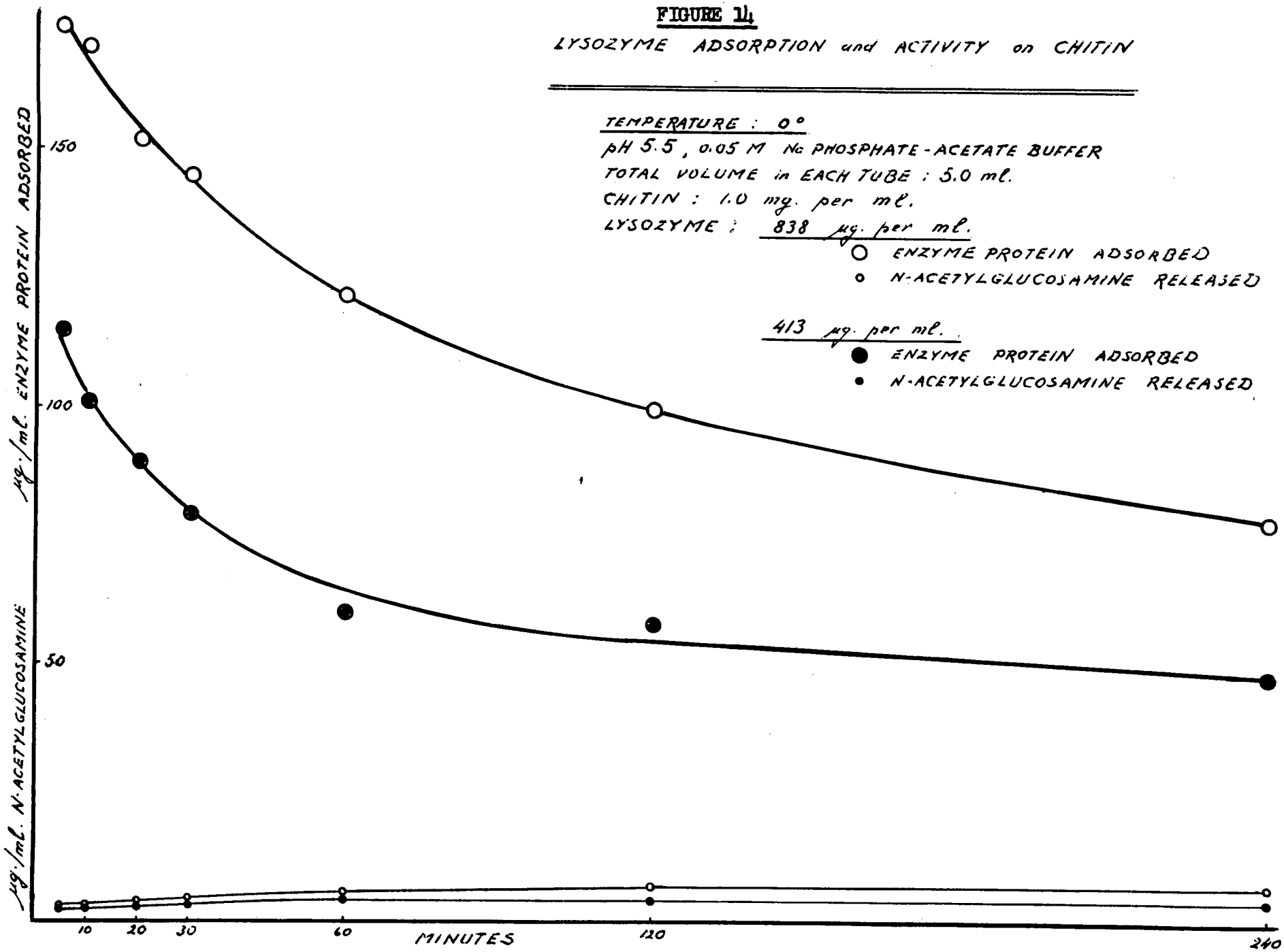


FIGURE 15

ADSORPTION and ACTIVITY of LYSOZYME on CHITIN

TEMPERATURE : 25°
pH 5.5 , 0.05 M Na PHOSPHATE-ACETATE BUFFER
TOTAL VOLUME in EACH TUBE : 5.0 mL.
CHITIN : 1.0 mg. per mL.
LYSOZYME : 842 µg. per mL.

○ ENZYME PROTEIN ADSORBED
○ N-ACETYLGLUCOSAMINE RELEASED

412 µg. per mL.

● ENZYME PROTEIN ADSORBED
● N-ACETYLGLUCOSAMINE RELEASED

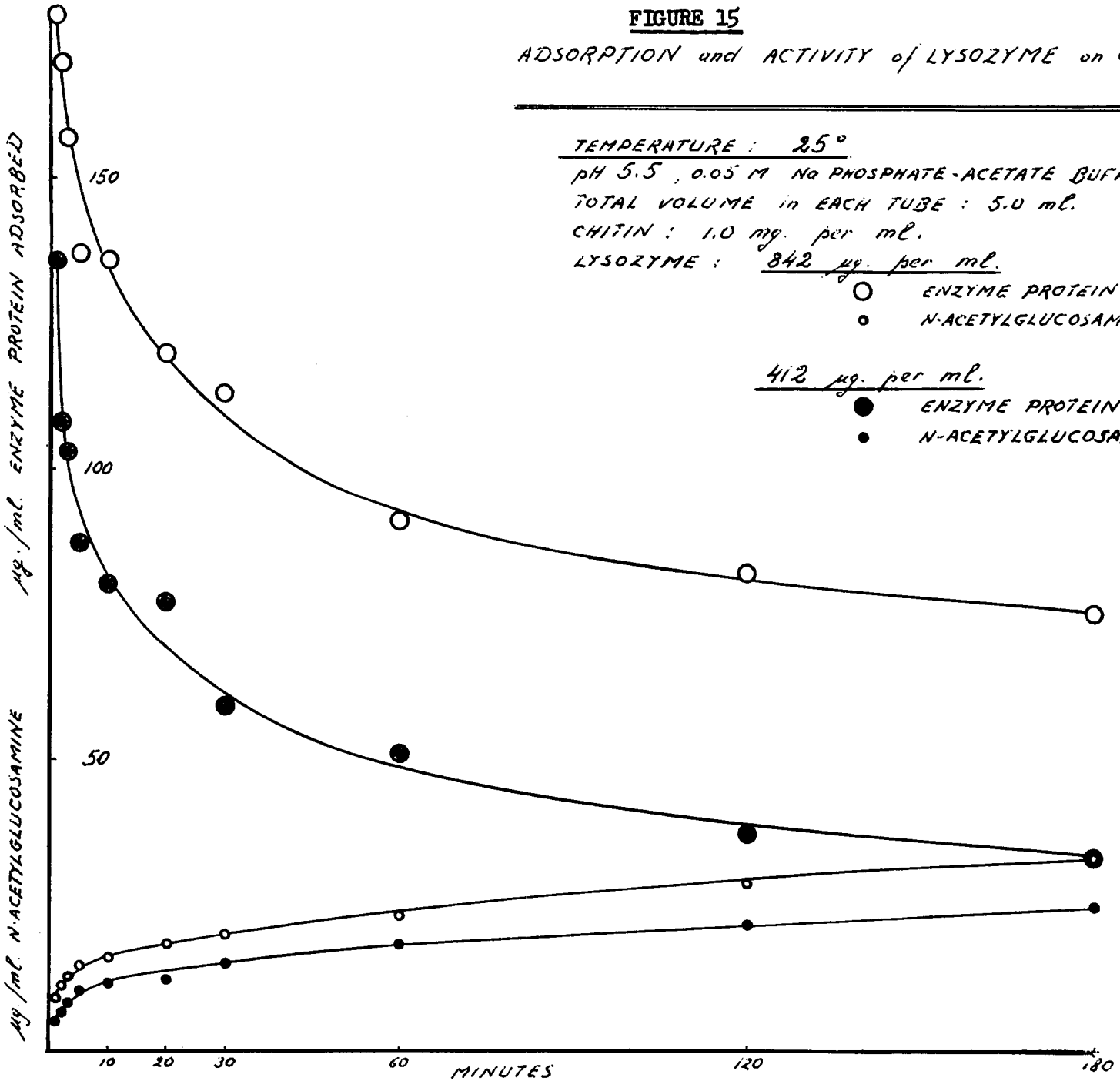


FIGURE 16

ADSORPTION and ACTIVITY of LYSOZYME on CHITIN

TEMPERATURE : 37°
pH 5.5, 0.05 M Na PHOSPHATE - ACETATE BUFFER
TOTAL VOLUME in EACH TUBE : 5.0 ml.
CHITIN : 1.0 mg. per ml.
LYSOZYME : 839 µg. per ml.

○ ENZYME PROTEIN ADSORBED
○ N-ACETYLGLUCOSAMINE RELEASED

415 µg. per ml.

● ENZYME PROTEIN ADSORBED
● N-ACETYLGLUCOSAMINE RELEASED

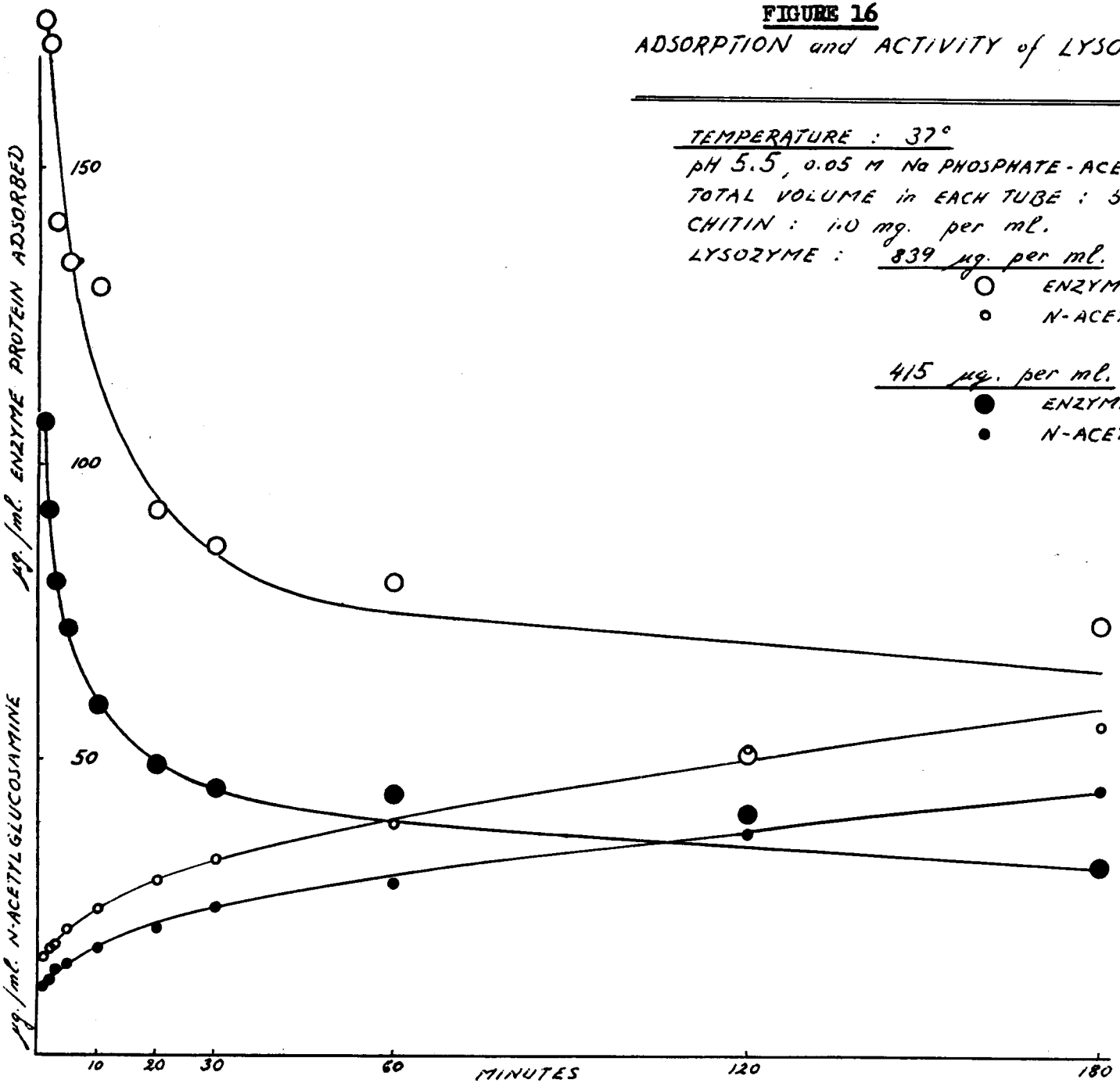
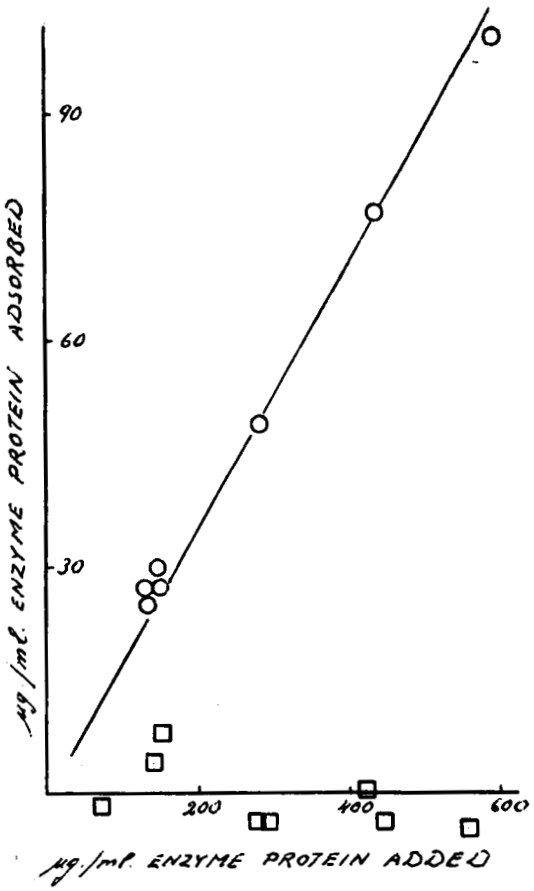


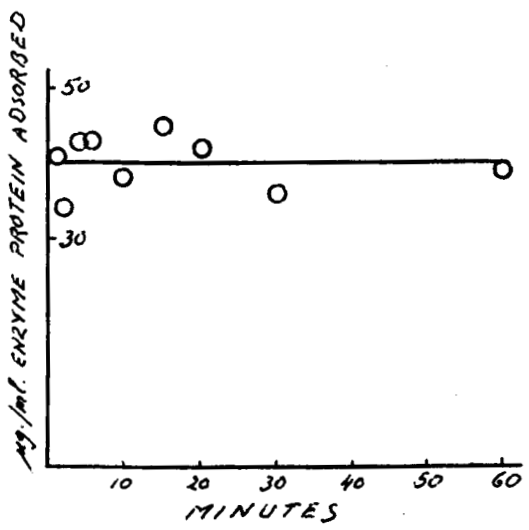
FIGURE 17



ADSORPTION of ACTIVE and HEAT DENATURED LYSOZYME on CHITIN

TEMPERATURE : 25°
CHITIN : 1.0 mg per ml.
pH 5.5, 0.05 M Na PHOSPHATE-ACETATE BUFFER
TOTAL VOLUME in EACH TUBE : 5.0 ml.
ADSORPTION TIME : 1 MINUTE
○ ACTIVE LYSOZYME
□ HEAT (100° - 10 HRS.) DENATURED LYSOZYME

FIGURE 18



ADSORPTION of CHITINASE on CHITIN

TEMPERATURE : 0°
CHITIN : 0.5 mg. per ml.
pH 5.5, 0.05 M Na PHOSPHATE-ACETATE BUFFER
TOTAL VOLUME in EACH TUBE : 5.0 ml.

D. Urease Activity in Media of Low Water Content.

The chemistry of the hydrolysis of urea by urease has been reviewed extensively in our previous reports (1, 2).

Although the naximum rate of the urease activity on urea is achieved in dilute aqueous solutions, our investigations have shown that the hydrolysis of urea by urease takes place also in concentrated and even saturated urea solutions at a measurable rate (ibid).

In order to explore further the kinetics of enzymatic reactions, specifically the hydrolysis of urea by urease at low moisture levels, we have initiated a study of the characteristics of this reaction at controlled atmospheric humidities.

Materials and Methods

Urea. Purified on a mixed bed ion exchange column and recrystallized as described (1). Stored in a desiccator under vacuum to minimize decomposition.

C^{14}-labelled urea was obtained from Calbiochem, Los Angeles. The C^{14}-urea was mixed with purified urea to obtain a stock containing 1 mc/g.

Urease. For the presently described experiments a non-crystalline urease preparation, 3 x NF, was used; obtained from Nutritional Biochemicals Corporation, Cleveland.

Apparatus. The radioactive gas counting chamber as described before (reference 1, Figure 5) was used. The increase of the $C^{14}O_2$ in the chamber was monitored with a Forro Company Geiger-Miller gas-flow tube (1) connected to a Nuclear-Chicago Company's scaler, and to a solid state count ratemeter, Lawrence Radiation Laboratory model

11 X 38F1 P-1, as described (3). The integrated amount of $C^{14}O_2$ in the chamber was strip-chart recorded on a Bausch and Lomb, hic., V.O.M.-5 recorder.

Method. 1 mg of C^{14}-urea, containing 1 μc C^{14} was mixed with 1 mg urease in a small glass container and placed in the radioactive gas counting chamber. The atmosphere in the chamber was equilibrated at the desired humidity before introducing the reactants. The relative humidity was maintained at the desired level throughout the experiment by placing a container containing appropriately diluted sulfuric acid in the chamber. The dilution of the sulfuric acid for the desired humidity levels (at $20^{\circ}C \pm 1^{\circ}$) was computed according to the International Critical Tables.

After it was established that no urea-urease reaction takes place below 60% relative humidity, 10 mg of C^{14}-urea were mixed with 1 mg of urease and placed in a desiccator as a stock mixture. 1.1 mg amounts of this dry stock were used for the rate determinations.

Results and Discussion

The results of the urease-urea-water reaction rates with 1:1 substrate-enzyme ratios are shown in Figure 19. At a 100% relative humidity at $20^{\circ}C$ the total maximum hydrolysis of urea was achieved in about 2 hours. An increasing rate of the $C^{14}O_2$ development during the first 30 minutes was evident at all humidity levels. It was apparent that this irregularity in the rate was caused by the water vapor adsorption on the components until the equilibrium conditions were reached. With decreasing humidity the rate decreased accordingly and there was no measurable $C^{14}O_2$ release at 60% relative humidity.

By decreasing the amount of available enzyme by an order of magnitude (1 mg C^{14}-urea + 0.1 mg urease) the reaction rates decreased, although the same total maximum value of urea hydrolysis was reached as before at 100% relative humidity, as shown in Figure 20. Similarly, there was no $C^{14}O_2$ development at a 60% humidity or below and the sigmoidal increase in the rate at the beginning of the reaction was evident.

Experiments are in progress to evaluate further the events taking place in this system, specifically, 1) to correlate the activity data with the hygroscopicity of individual components, i.e., whether the water vapor adsorption on the enzyme or on the substrate is the limiting factor in this reaction; 2) how much water becomes available to the reactants quantitatively at discrete reaction rates, i.e., at discrete humidities; 3) the kinetics of the urease denaturation at these conditions, and the manner in which such an inactivation of urease influences the total $C^{14}O_2$ development rate.

Summary

The examination of the hydrolysis of urea by urease at a limited water availability was continued. It was shown that urea placed in a 100% humidity atmosphere was hydrolyzed by urease to completion, whereas there was no reaction at 60% relative humidity and below.

References

1. Falkinham, J. O., Skujins, J. and McLaren, A. D. First Semiannual Progress Report, Space Sciences Laboratory, University of California, Berkeley, March 9, 1965; NASA-CR-57585.

2. Falkinham, J. O., Skujins, J. and McLaren, A.D., Second Semiannual Progress Report, Space Sciences Laboratory, University of California, Berkeley, July 20, 1965; NASA-CR-64268.

3. Skujins, J. J. and McLaren, A. D., Fourth Semiannual Progress Report, Space Sciences Laboratory, University of California, Berkeley, July 12, 1966; NASA-CR-76549.

FIGURE 19

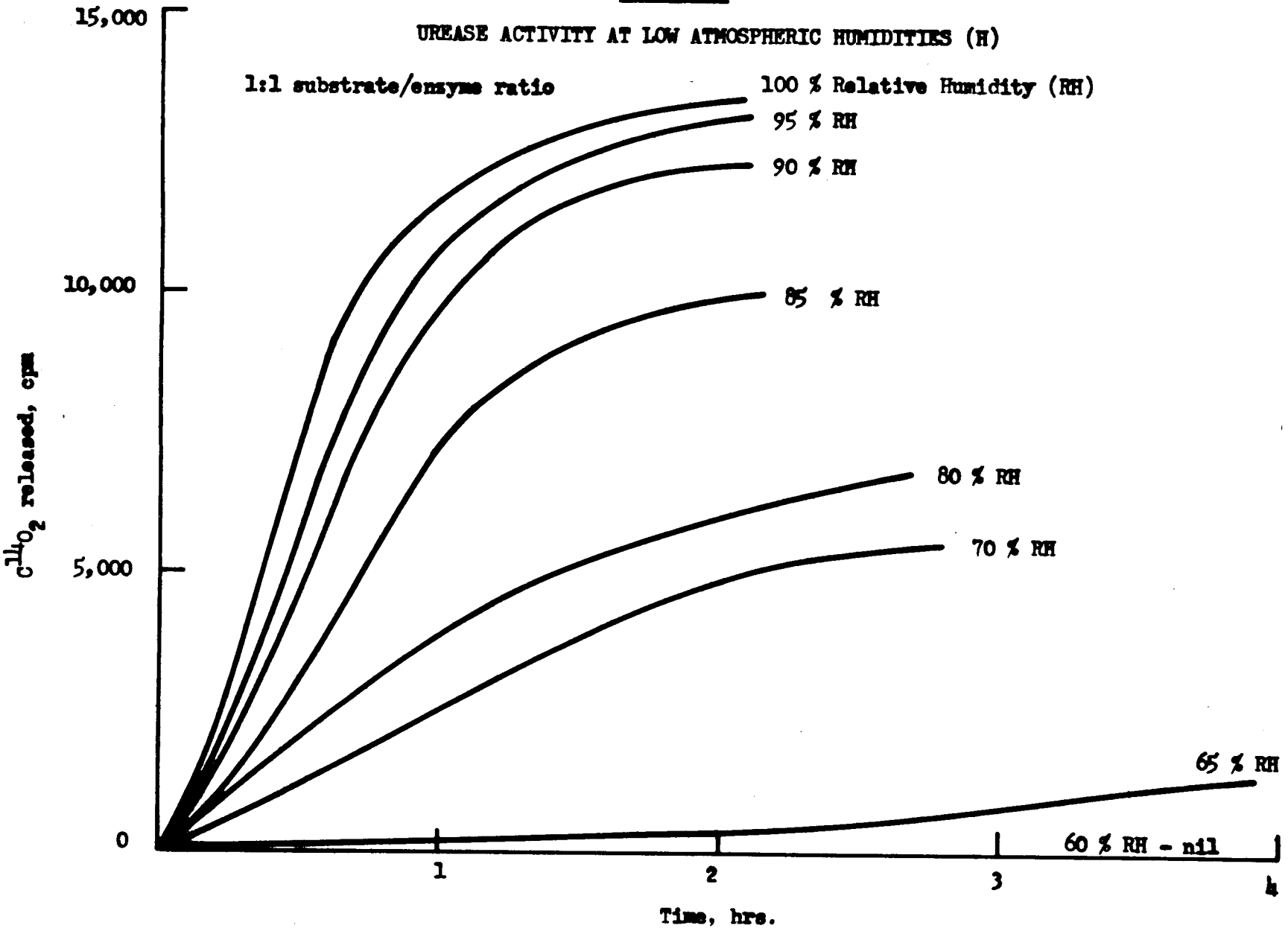

UREASE ACTIVITY AT LOW ATMOSPHERIC HUMIDITIES (H)
1:1 substrate/enzyme ratio
100 % Relative Humidity (RH)
95 % RH
90 % RH
85 % RH
80 % RH
70 % RH
65 % RH
60 % RH - nil
15,000
10,000
5,000
0
$C^{14}O_2$ released, cpm
1
2
3
4
Time, hrs.

FIGURE 20

UREASE ACTIVITY AT LOW ATMOSPHERIC HUMIDITIES

10:1 substrate/enzyme ratio